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13. ABSTRACT <i>(Maximum 200)</i> Recent evidence has shown that hepatocyte growth factor (HGF) is upregulated in human breast cancer, and that this high level of expression is a reliable indicator of recurrence and of poor overall survival of breast cancer patients. We have recently demonstrated co-expression of HGF and HGF receptor (Met) mRNA in invasive human breast carcinomas, as well as in regions of benign hyperplasia. In contrast, most nonmalignant epithelia express Met, but not HGF. These observations suggest that aberrant expression of HGF and subsequent establishment of an autocrine HGF loop could lead to abnormal growth, tumorigenesis and metastasis. The objectives of this grant are: a) to assess expression of HGF and Met in nonmalignant and malignant breast epithelia, b) to examine the transcriptional and post-transcriptional mechanisms of regulation of HGF mRNA and protein, and c) to determine if upregulating, or downregulating HGF or Met expression affects the transformed and tumorigenic phenotypes of mammary carcinoma cells <i>in vitro</i> and <i>in vivo</i> . We have shown that Met mRNA was expressed in seven mammary carcinoma cell lines examined, and Met protein was expressed in all but one cell line. HGF mRNA and active HGF protein were expressed in two of seven carcinoma cell lines. Two additional carcinomas expressed putative HGF protein which showed no activity. Conditions that regulate transcriptional and post-transcriptional expression and processing of HGF are currently being examined. In addition, antisense oligonucleotide strategies have been developed to assess the role of HGF and Met in growth and metastasis of breast carcinomas.			
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FOREWORD

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Bruce E. Elliott, October 10, 1997
PI - Signature Date

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5. INTRODUCTION:

Background: Hepatocyte growth factor (HGF) has been shown to be up-regulated in human breast cancer (1), and recent evidence suggests that this high level of expression is a reliable indicator of recurrence and of poor overall survival of breast cancer patients (1). The HGF receptor gene, *MET*, is frequently over-expressed or amplified in many types of human cancers including ovarian (2) and colorectal (3) carcinomas, melanomas (4), and osteosarcomas (5). In some cases constitutive activation of HGF receptor (Met) was also observed (5). These observations have suggested that sustained activation of the Met signal transduction pathway may be important for development of cancers through autocrine or paracrine mechanisms. This view was strongly supported by the fact that transfection of a full length *MET* cDNA can induce a tumorigenic and metastatic phenotype upon establishment of an HGF autocrine loop in NIH 3T3 fibroblasts (6,7) and C127 cells (8). In addition, diverse tumorigenesis and aberrant development occurs in transgenic mice over-expressing HGF (9). In contrast, HGF expression in normal epithelial cells is strongly suppressed by tissue specific repressors (10).

Our laboratory (11), and others (12,13), have recently demonstrated co-expression of HGF and Met mRNA in primary human breast carcinomas, as well as in regions of benign ductal hyperplasia (see Figure 1). These findings suggest the possibility that abnormal expression of HGF protein occurs in certain mammary carcinomas, and that establishment of an HGF autocrine loop could lead to abnormal growth, tumorigenesis and metastasis. Recent reports have shown that HGF and Met are co-expressed during early development before reciprocal expression in adjacent tissues during morphogenesis (14), as well as in some specific cell types, e.g. undifferentiated myoblasts (8) and proliferating endothelial cells (15). Also, expression of a constitutively active Met kinase in a myogenic C2 cell line inhibits myogenic differentiation (8). Thus paracrine and autocrine activation of Met may have opposing effects during development, whereas an abnormal switch to autocrine activation of Met in epithelial cells may promote a malignant and metastatic phenotype. However, the natural occurrence of an HGF autocrine loop in breast cancer and the functions involved are yet to be determined.

Previous work in this laboratory: To assess the putative role of HGF in stromal-tumor interactions in breast cancer, we examined both paracrine and autocrine HGF functions in an established murine mammary carcinoma cell line, SP1. We showed that adipocytes, an important stromal cell type, secrete HGF which stimulates increased growth and invasiveness of SP1 cells (16). In addition, SP1 cells, and metastatic variant SP1-3M cells derived from SP1, express mature HGF and phosphorylated Met, suggesting the existence of an HGF autocrine loop. Additionally, SP1 cells exhibited spontaneous anchorage-dependent growth in serum-free medium and invasion through matrigel-coated transwell membranes. Phosphorylation of Met and spontaneous invasion of SP1 cells was inhibited by neutralizing anti-HGF antibody (17). Furthermore, we have demonstrated that phosphatidyl inositol (PI) 3-kinase is constitutively tyrosine-phosphorylated in SP1 cells, and that activation of PI 3-kinase is required for HGF-induced autocrine growth (18) and survival (data not shown) of SP1 cells. Together, these findings suggest that both paracrine and autocrine stimulation by HGF can occur in SP1 carcinoma cells. Whereas paracrine regulation by stromal cells may be

important in normal epithelial development, an HGF autocrine loop leading to sustained activation of the Met pathway may be important in the development and progression of breast carcinomas.

The following working **HYPOTHESIS** forms the basis of the present USAMRMC-supported programme. Whereas nonmalignant epithelial cells do not express HGF, we have shown abnormal co-expression of HGF and Met in human breast carcinomas (11) and in a murine mammary carcinoma cell line (Figure 1). Based on these findings, our hypothesis is that co-expression of HGF and Met results in the establishment of an HGF autocrine loop which provides a selective advantage for autonomous growth and metastasis of mammary carcinoma cells. Regulation of expression of functionally-active HGF in mammary epithelial cells could occur at several levels from transcription (10) to post-translational modification by enzymatic processing (19). The following predictions can be made from this hypothesis: Conversion from paracrine to autocrine HGF expression by up-regulation of HGF expression would be expected to enhance certain HGF-dependent functions and to promote the tumorigenic phenotype. Inhibition of an existing HGF autocrine loop by down-regulation of HGF or Met mRNA expression or blocking of HGF protein maturation, would be expected to abrogate HGF-dependent functions and to suppress tumorigenic properties. In view of the pleiotropic functions of HGF, the affect of a sustained HGF autocrine loop on nonmalignant and malignant epithelial cells may differ, depending on the stage of progression.

MATERIALS AND METHODS: Breast carcinoma cell lines used in this study are described in Table I. Details of materials and methods are described in publications referenced in the Results section. See also Figure legends.

6) RESULTS:

Objective (I): To assess secretion and maturation of HGF and Met expression in nonmalignant and malignant breast epithelial cells, and to correlate with HGF-induced cellular functions:

Recent results from our laboratory (Figure 1) (11) and Wang *et al.* (12) have shown that HGF mRNA is expressed in human invasive breast carcinoma cells as well as in regions of ductal epithelial hyperplasia. Preliminary results also showed immunoreactive HGF protein associated with carcinoma cells in human breast tissues (Tuck *et al.*, unpublished result). However, exogenous HGF protein could be bound to, or endocytosed by, these carcinoma cells. Furthermore, it is not known from these studies whether post-translational processing of pro-HGF occurs in benign or malignant epithelial cells from primary breast tissues. Recently, the HGF- and Met-related family members, macrophage stimulating protein (MSP) and Ron, respectively, have been shown to be expressed in a complementary manner to HGF and Met during mammary tumor progression (Giordano, personal communication), suggesting different regulatory functions of these growth factors. We therefore are examining the expression of HGF, Met, MSP and Ron mRNA and protein in primary human breast tissues and in newly-established nonmalignant and malignant breast epithelial cell lines.

Mammary tissues and cell lines: For these studies, we are using carcinoma cells purified from human breast tumor tissues and various newly derived nonmalignant and malignant breast epithelial cell lines (see Table I). We are currently using purified mammary stromal and epithelial cell types from non-malignant and malignant breast tissues, prepared by Dr. J. Emerman (Dept. of Anatomy and Cell Biology, U.B.C., see attached letter). These purified populations of stromal and breast epithelial cells have proven to be excellent *in vitro* correlates for the study of stromal-epithelial cell interactions in human breast (20,21). In this way we will confirm whether HGF and Met are expressed in stromal and epithelial cells freshly isolated from human breast cancer specimens.

In addition to primary human breast tissues, we are also examining various nonmalignant and malignant breast epithelial cell lines (see Table I). Previously, we demonstrated that a murine mammary carcinoma, SP1, expressed a 6 kb HGF mRNA, and secreted an 82 kDa HGF protein, corresponding to the reported sizes of HGF mRNA and mature protein, respectively (17). A similar approach is being used to examine HGF and Met expression in three mammary epithelial cell lines and corresponding malignant cell lines derived from them: MCF10A1 (human, obtained from H. Soule (22)), 21PT (human, obtained from R. Sager (23)), and TM3 (mouse, obtained from D. Medina (personal communication)) cells. MCF10A1 cells are a subclone of a transformed (non-tumorigenic) cell line derived from a human subcutaneous breast mastectomy. MCF10A1 cells were transfected with the *Ha-ras* oncogene to yield a cell line, MCF10A1neoT (24). MCF10A1neoT forms tumors in nude mice, from which a cell line, MCF10AT3B, was established. 21PT cells are a nonmalignant transformed breast epithelial cell line derived from a patient with infiltrating mammary carcinoma (23); 21NT (tumorigenic) and 21MT (metastatic) carcinoma cells are derived from the same patient. TM3 cells are a Balb/c mouse-derived mammary epithelial cell line from which a malignant breast epithelial cell line, T-2410L TM6, was established (Medina, unpublished). Two other newly established human breast carcinoma cell lines are also being studied (WO-E, EL-E, obtained from Dr. B. Campling, Dept. Oncology, Queen's U.). Both RT-PCR and western blotting approaches have been developed for detection of HGF and Met in the above tissues and cell lines.

RT-PCR analysis of HGF and Met mRNA in mammary carcinomas: To detect expression of putative HGF and Met mRNA and protein in nonmalignant and malignant breast epithelial cells, we have initiated RT-PCR analysis. Primers were designed to specifically detect *HGF*, *MSP*, *MET* and *RON* cDNA, and to recognize the corresponding cDNAs of mouse and human (Figure 2). Primers specific for the house-keeping genes, transferrin and β -glucuronidase (25), are being used as controls to relate the relative amounts of PCR products detected with physiological levels of expression in the same mRNA samples. For reliable quantitation of product, PCR conditions must be selected so that quantitation is performed in a linear phase of amplification, where concentrations of product are proportional to starting levels of target. Preliminary experiments were conducted to determine the kinetics of amplification over a range of cycle numbers to select conditions for which amplification was linear. Quantification was carried out by directly measuring intensity of ethidium-bromide stained gels or by using end-labelling of the forward primer in the PCR and measuring the amount of radioactive incorporation. Template *HGF* and *MET* cDNAs from plasmids or transcribed from RNA from SP1 cells were amplified for a range of cycles from 5 to 33 times. A linear range of amplification was found between 18 and 30 cycles (Figure 3). Experiments are in

progress to ensure equal efficiency with different amounts of starting template. In the PCR experiments reported here, we used 25 cycles.

Our results showed that 7/7 mammary carcinoma cell lines expressed Met mRNA (Table I). In contrast only 2/7 mammary carcinoma cell lines (SP1 and SP1-3M) expressed HGF mRNA. Recently, non-small cell lung carcinomas (NSCLC) have been shown to express HGF mRNA and protein (26). Therefore, to provide a comparison with another carcinoma cell type, we have also examined HGF and Met mRNA expression in a series of NSCLC. Our results showed that 8/8 NSCLC cell lines expressed Met mRNA; whereas only 1/6 NSCLC cell lines expressed HGF mRNA. Representative results are shown in Figure 4 and Table I. The remaining cell lines in this series are currently being examined.

Analysis of HGF protein in mammary carcinomas: HGF protein was examined in conditioned media of the various cell lines tested. Copper (II)-immobilized affinity chromatography, a method developed in this laboratory by a Ph.D. student, Nader Rahimi, to purify HGF (Rahimi *et al.*, 1996), was employed. The principal of separation of HGF from biological samples by copper (II) affinity chromatography is based on the fact that HGF has several cationic sequences (His-X-His) in the kringle domains of the HGF molecule (27). The copper (II)-immobilized affinity column was prepared by adding CuSO₄ to a pre-packed column of iminodiacetic acid coupled to epoxy-activated sepharose. HGF was specifically eluted from the column by increasing amounts of iminodiacetic acid. Figure 5B illustrates the eluate from the column using CM from HEL299 cells (a human embryonic fibroblast cell line that expresses HGF), as a positive control for HGF compared to 100 ng of rHGF. This approach was used to detect HGF protein in CM from human mammary epithelial cells (MCF10A1) and carcinoma cells EL-E (Figure 5A), as well as SP1 and SP1-3M murine mammary carcinoma cells (see Ref. (17, 69)); no HGF protein was detected in CM from WO-E breast carcinoma cells. rHGF, and HGF purified from HEL299 CM are shown again in Figure 5C, with putative HGF protein purified from CM from BH-E (a NSCLC cell line) (Figure 5D and Figure 6). In summary, putative HGF protein was found in five of eight mammary carcinoma cell lines, and one of three nonmalignant epithelial cell lines tested. In addition, one (BH-E) of eight NSCLC cell lines expressed putative HGF protein. Interestingly, one NSCLC cell line (SKLC-6) expressed significant HGF mRNA (Figure 4D), but no detectable HGF protein. Experiments are in progress to examine the activity of putative HGF protein in mammary carcinoma cells (Objective II).

Analysis of Met protein in mammary carcinomas: To aid in the determination of Met expression in the various cell lines, rabbit antibodies to human Met were developed. A glutathione S-transferase (GST)-fusion protein was made using an amino acid fragment from the carboxyl-terminus of Met (Val₁₂₉₈ - Ser₁₃₉₄) (Figure 7), which was expressed by bacteria, purified and then injected into two rabbits. The antibodies produced (MET1 and MET2) were characterized for their potential use in immunoprecipitation, immunoblotting, crossreactivity with other species of Met, and immunohistochemistry using cell lines that express Met. It was determined that the antibodies could immunoprecipitate Met by incubating whole cell lysates of A549 cells with MET1 and MET2 antibodies (up to 1/200 dilution). However, neither antibody could be used for immunoblotting (data not shown). It was also determined that MET1 and MET2 were not crossreactive with mouse HGF

(data not shown). Our results showed that all mammary epithelial and carcinoma cell lines tested expressed Met, except one carcinoma cell line (WO-E) (Figure 8B and Table I). Four of eight NSCLC cell lines (Figure 8C) examined showed detectable Met protein. Interestingly, the remaining four NSCLC cell lines showed detectable Met mRNA (see above), but no Met protein. This point is addressed in the discussion.

Maturation status of putative HGF protein secreted by mammary carcinoma and non-small cell lung carcinoma cell lines: HGF is secreted as an inactive single-chain pro-HGF protein of 105 kDa (28). Pro-HGF is cleaved by serine proteases at Arg₄₉₄-Val₄₉₅ and is converted to a heterodimeric mature HGF molecule consisting of disulfide-linked α (65 kDa) and β (30 kDa) chains of 463 and 234 amino acid residues respectively (19). Processing of HGF is required for biological activities of HGF. Examples of serine proteases capable of activating HGF are urokinase plasminogen activator (29), a protease homologous to blood coagulation Factor XII (30), HGF converting enzyme (31), and other related HGF activating proteases (32). In addition, activity of HGF can be affected by association with ECM proteins, such as heparin (33).

Three approaches are being taken to assess the maturation status and activity of HGF secreted by carcinoma cells. First, copper(II) affinity-purified HGF from CM was subjected to SDS-PAGE under reducing conditions, in which case α and β chains of HGF should dissociate. Figure 6 shows a comparison between reduced and non-reduced HGF from HEL299 CM and rHGF (positive controls), and putative HGF protein purified from BH-E CM. A shift to lower M_r (approx. 80 kDa) of the band immunoreactive with anti-HGF antibody is seen when rHGF and HEL299 CM are reduced, corresponding to the dissociated α -chain of HGF. In addition, a partial shift in the positions of the bands immunoreactive with anti-HGF antibody in BH-E CM was observed, however the identity of the lower molecular weight band as the HGF α chain is not yet confirmed. An anti-human HGF monoclonal antibody recently provided by Genentech is being used to confirm the HGF identity of this band. Analysis of CMs from other cell lines (e.g. EL-E, and MCF10A1) is currently in progress. A notable similarity among all of the CM tested, was the presence of several high molecular weight bands (approximately 200 kDa and greater) detected by immunoblotting, possibly due to complexes of HGF formed during purification.

Second, we examined whether Met was tyrosine phosphorylated on carcinoma cells, indicating auto-activation. Our results showed no detectable phosphorylation of Met on EL-E or MCF10AT3B mammary carcinoma cells, although Met in these cells could be activated with rHGF or an activating anti-Met antibody (data not shown).

Third, CM from carcinoma cell lines was incubated with A549 cells (Met positive, HGF negative) for 30 min to determine if Met on A549 cells could be activated, as determined by tyrosine phosphorylation. A control showed strong tyrosine phosphorylation of Met in A549 cells incubated with HGF (40 ng/ml) (Figure 9). Figure 10C shows that EL-E and MCF10A1T3B CMs do not activate Met on A549 cells under conditions in which the same cells can be activated by incubation with rHGF (positive control). Similar experiments are in progress to examine HGF activity of BH-E CM. Thus, putative HGF protein in mammary carcinoma cell lines EL-E and MCF10AT3B has no

detectable HGF activity. This work has been carried out primarily by Jin Gui (Ph.D. student) and Jennifer Klassen (M.Sc. student) in my laboratory.

Objective (II): To examine the regulation of HGF mRNA expression and maturation of HGF protein in nonmalignant and malignant mammary epithelial cells *in vitro* and *in vivo*:

Effect of cytokines, growth hormones and microenvironment on HGF mRNA and protein expression: Cytokines and growth hormones such as TPA, TNF- α , IL-1, IL-6 (34,35), and estrogens (36,37) can stimulate HGF expression, whereas TGF- β inhibits HGF expression in fibroblasts (10). We have shown that estrogen stimulates *in vivo* growth and metastasis of some mammary carcinomas (39), consistent with the possibility that estrogen may induce HGF expression in these cells. Recently, a novel transcriptional regulatory region within the core promoter of the HGF gene was found to regulate HGF inducibility by cytokines via the C/EBP family of transcription factors (38). Hypoxia-induced factor-I (HIF-I) binding sites and shear-stress response elements (SSRE) are also found in the HGF promoter, suggesting a possible role of HGF response to stress such as lack of oxygen and shear-stress (Figure 11). Both HIF-I binding sites (40) and SSRE (41) are involved in the transcriptional regulation of expression of a variety of growth factors including vascular endothelial growth factor (VEGF), platelet-derived growth factor-B (PDGF-B), and TGF- β . These various conditions will be tested for their ability to modulate HGF expression in nonmalignant and malignant breast epithelial cell lines (Table 1).

Other physiologically relevant conditions are being used to examine the pattern of HGF expression in epithelial cells. One approach is the use of 3-dimensional culture conditions which have been shown to be useful for normal and malignant breast tissue. The 3-D reconstituted basement membrane, Matrigel, promotes growth and morphogenesis of normal epithelial cells which form organized acini similar to those in normal breast tissues, whereas malignant cells form disorganized, invasive colonies comparable to cells in primary tumors (20,21). Another approach involves the role of ECM proteins, which regulate a wide variety of tissue specific genes, in regulating expression of HGF and Met.

The effect of the above conditions on HGF and Met expression in mammary epithelial and carcinoma cells will be examined using RT-PCR and HGF protein analyses described in Objective I. These experiments will provide a basis to study transcriptional and post-transcriptional regulation of HGF expression in mammary tumor progression, which will be an important aim in the next year of this grant period. This work is being carried out by Wesley Hung, who joined my laboratory as a postdoctoral fellow in July 1997.

Role of Met and downstream signalling molecules, Ras, PI 3-kinase and c-Src, in HGF expression in malignant cells: Since an autocrine HGF loop has been demonstrated in some mammary carcinoma cells (Objective I), and the activity of HGF may be necessary for the survival and growth of these cells, it is possible that HGF might activate its own expression through the activation of Met, as has been shown for auto-regulation of Met (42). We are therefore examining the effect of Met and downstream signalling molecules, Ras, Src and PI 3-kinase (43) on HGF

expression in mammary carcinoma cells. In a parallel project, supported by a grant from the Canadian Breast Cancer Research Foundation, we have recently shown that Src kinase is activated in SP1 carcinoma cells expressing HGF and Met, and that Src kinase activity is required for anchorage-independent growth of these cells (unpublished). We have also shown that PI 3-kinase is required for HGF-induced growth (18) and survival (unpublished) of carcinoma cells. In addition, activation of Ras is required for some HGF functions (e.g. motility (44)), and Ras (45) as well as Src (46) are known to regulate the expression of VEGF, which regulates angiogenesis. Therefore, we are examining the effect of Met and downstream signalling molecules, Ras, Src and PI 3-kinase, on expression of HGF in carcinoma cells.

To determine if Met activation is important in HGF expression, we will assess HGF mRNA and protein levels in SP1 carcinoma cells in which Met expression has been depleted by antisense oligonucleotides specific for Met mRNA (Objective III). Likewise, the development of carcinoma cell populations defective in downstream signalling pathways, Ras, Src and PI 3-kinase, is being carried out as part of a project supported by the Canadian Breast Cancer Research Foundation. To modulate activity of specific signalling molecules, two approaches are being used. First, we are using pharmacological inhibitors of tyrosine kinases (e.g., herbimycin A), Ras-related proteins (B956) (47,48), and PI-3 kinase (LY294002) (49,50)). In preliminary results, we demonstrated that treatment of SP1 cells with herbimycin A, B956 and LY294002 inhibited proliferation of these cells without affecting cell viability (Figure 12), thus confirming the optimum conditions of these pharmacological agents. Herbimycin A also inhibited Src kinase activity in SP1 cells (unpublished result). Activities of Ras and PI 3-kinase in cells treated with the corresponding inhibitors are being tested. Second, we are expressing dominant negative mutant cDNAs for Ras (RasN17 (51)), Src (Src-RF, provided by J. Brugge), and PI 3-kinase (Δ p85 (52)) in SP1 cells. Previously we have shown that expression of Src-RF and Δ p85 in SP1 cells repressed the corresponding kinase activity, and reduced the ability of these cells to form colonies in soft agar (unpublished result). These cell populations will be examined for HGF mRNA and protein levels using RT-PCR and western blotting analysis as described in Objective I. This work is being carried out by Wesley Hung in my laboratory.

Identification of HGF-activating proteases in mammary carcinomas: The proteases responsible for HGF processing in breast carcinoma cells have not yet been identified, and the activity of these proteases may directly affect the tumorigenic activity of HGF. As a first step to identifying proteases involved in HGF maturation in breast carcinoma cells, we are developing an assay for the detection of enzymes capable of cleaving pro-HGF at the Arg₄₉₅-Val₄₉₆ site (31). We have constructed a GST-fusion protein (53) consisting of GST linked to an HGF peptide comprised of amino acids flanking the cleavage site (Figure 13). The uncleaved GST-HGF protein has a M_r of 33 kDa, which is reduced to 28 kDa following cleavage. We have been successful in expressing the GST-HGF fusion protein in bacteria, however upon purification it appears to be present primarily in the cleaved state (data not shown). We are currently investigating whether other fusion proteins (e.g. consisting of maltose binding protein (54)) would be preferable to GST. This system will be used to screen CM from mammary carcinoma cells for HGF-activating proteases. CMs showing proteolytic activity will then be used to purify the putative protease(s) using a combination of ion exchange, size exclusion and affinity chromatographic procedures, as proposed in this USAMRMC grant. This work is being carried out by Wesley Hung, a postdoctoral fellow in my laboratory.

Objective (III): To determine if up-regulating or down-regulating HGF mRNA and protein expression in nonmalignant and malignant mammary epithelial cells affects the transformed and tumorigenic phenotypes of these cells *in vitro* or *in vivo*.

Antisense oligonucleotides as a tool for down-regulating HGF and Met mRNA and protein expression in mammary carcinoma cells: The use of anti-sense RNA and antisense oligonucleotides specific for several growth factor receptors and ligands including epidermal growth factor receptor (55), insulin-like growth factor-1 receptor (56), basic fibroblast growth factor (57), and PKC- α (58) have been successfully used to down-regulate the expression of corresponding target proteins thereby disrupting their biological functions. We have initiated a collaboration with Dr. R. Wagner (Gilead Sci.), to develop antisense oligonucleotides to HGF and Met mRNA. We expect that these antisense oligonucleotides will down-regulate HGF and Met protein expression, thereby disrupting the HGF autocrine loop in SP1 (mouse) and MCF10AT3B (human) carcinoma cells, which express and secrete HGF protein. Since HGF is a secreted protein, the intracellular pool size of mature HGF is low, and therefore this system is highly amenable to down-regulation by antisense strategy. Dr. R. Wagner has synthesized oligonucleotides predicted to have antisense activity directed against the human and mouse HGF and Met mRNA (Figures 14,15,16), as well as corresponding mismatched oligonucleotide controls. Oligonucleotides (15 mer) were synthesized to target specific coding regions within the gene, and were modified to include a phosphorothioate backbone to increase stability. In addition, they were selected to have the following characteristics: non-toxicity, no self-complementarity, higher than average thermal melting point (Tm), and high affinity for target RNA.

For biological studies, antisense oligonucleotides are being delivered into epithelial and carcinoma cells using a lipid, Cytofectin (59). Preliminary results with a FITC-conjugated oligonucleotide (15 mer) showed incorporation of oligonucleotides into virtually all carcinoma cells treated, and into the nuclei of approximately 60-70% of these cells (Figure 17). Six antisense oligonucleotides inhibited Met protein expression in SP1 cells by 30-50%, as determined by western blotting (data not shown). Further experiments comparing the effect of specific versus mismatched oligonucleotides on HGF mRNA and protein expression are in progress. Proliferation of SP1 carcinoma cell lines, which co-express HGF and Met, in low-serum medium is being used as an initial functional read-out. Preliminary results (Figure 18) show a specific reduction in proliferation following treatment of SP1 cells with one antisense oligonucleotide to Met, compared to an unrelated control oligonucleotide, consistent with a role of autocrine activation of Met in the spontaneous proliferation of these cells. In the next phase of this work, the effect of down-regulating Met or HGF will be tested in various functional assays, eg. anchorage-independent growth, motility and invasion. This work is being carried out by Jin Gui, a Ph.D. student in my laboratory.

7) CONCLUSIONS:

In summary, we have demonstrated that: a) Met mRNA is expressed in all mammary epithelial and carcinoma cell lines tested, and all but one cell line (WO-E) expressed detectable Met protein; b) HGF mRNA and active HGF protein are expressed in two (SP1 and SP1-3M) of seven mammary carcinoma cell lines tested; c) two additional mammary carcinomas (EL-E and MCF10A.T3B), and one of six NSCLC expressed putative HGF protein which showed no activity as determined by ability to stimulate tyrosine phosphorylation of Met. A manuscript on this work is currently being prepared. These experiments relate to Tasks 1, 2 and 5 in this USAMRMC grant.

The above results indicate that the majority of mammary carcinoma and NSCLC cell lines do not secrete detectable HGF protein and that those which do express putative HGF protein show no evidence of autocrine HGF activity. In contrast, our previous results had shown that HGF and HGF mRNA are co-expressed in mammary carcinomas, particularly in regions of invasive carcinoma. In addition, Olivero *et al.* (60) have shown overexpression and activation of HGF in human NSCLC samples. Our results therefore emphasize the importance of *in vivo* tissue microenvironment in transcriptional and post-translational mechanisms regulating HGF expression. Based on our results, at least three possible effects may occur in carcinoma cells: 1) both HGF mRNA and HGF protein are absent, indicating transcriptional shut-down of the HGF gene; 2) HGF mRNA is present but no secreted HGF protein is detected, suggesting defective translational or post-translational stages of HGF expression, and 3) HGF protein is secreted but is not active, suggesting lack of proteolytic enzymes required for cleavage, or presence of inhibitors of HGF function. Studies are in progress to examine the mechanisms of transcriptional and post-translational regulation of HGF expression in the above cell systems. Of particular interest, are possible post-transcriptional mechanisms regulating RNA stability (61) or translation (62). In addition, we are examining conditions that regulate post-translational processing of HGF in carcinoma cells.

Our results suggest that HGF protein may be produced in some mammary carcinomas, but may remain inactive due to lack of appropriate serine proteases required for cleavage of pro-HGF. To test for HGF-activating enzymes in CM, we are developing an assay involving a GST-HGF fusion protein. The principal of the assay is that active serine proteases would cleave the GST-HGF fusion protein resulting in a protein of reduced M_r , easily detectable by SDS-PAGE. Technical aspects of this assay are still being developed. CMs showing HGF-activating proteases will be used to isolate and further characterize candidate proteases as described in the grant. We suspect that members of the urokinase plasminogen activator (uPA) family may be involved, since expression of the serine proteases, uPA and uPA receptor, is upregulated in some carcinomas in response to HGF, and these proteases are capable of activating HGF (63-65). This study relates to Task 5 of the present grant.

In the next phase of this study, we will examine HGF and Met expression in purified mammary epithelial and carcinoma cells from human breast tissue samples to confirm the status of HGF and Met expression and activity *in vivo*. We are continuing to assess the role of the tissue microenvironment by examining the affects of cytokines, ECM proteins, 3-dimensional growth conditions and hypoxia on HGF and Met expression and activity. These conditions in part reflect the *in vivo*

microenvironment of mammary tumors, and have been shown to be important in regulating mammary tumor progression (21,66-68).

Finally, preliminary experiments using antisense oligonucleotides specific for HGF and Met mRNA indicate that this approach may be successful in downregulating the expression of the corresponding proteins in mammary carcinoma cells. Recent studies with transgenic mice have shown that knock out of HGF or Met severely impedes liver development and causes death *in utero* (70). However, to date attempts to knock out HGF or Met in carcinoma cells have not been successful, possibly due to the polyploidy and genetic instability of malignant cells. Antisense strategy therefore remains an important approach in studies of HGF and Met in tumor progression.

Information from these studies may provide new insights into the events involved in de-regulation of HGF in breast carcinoma cells, and may provide putative new targets, at the transcriptional or post-translational stages of HGF expression and maturation, for inhibitor designs in the treatment of breast cancer (Figure 19).

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Table 1**Expression of HGF and Met in human and breast epithelial and carcinoma cell lines**

Breast Cell Line	Species	Malignant Status	HGF Secretion		Met Expression	
			RNA	Protein	RNA	Protein
WO-E ^a	Human	+	-	-	+	-
EL-E ^a	Human	+	N/D	+- ^f	+	+
MCF10A1 ^b	Human	-	N/D	+-	N/D	+
MCF10A1neoT ^b	Human	-	N/D	+-	N/D	+
MCF10A1T3B ^b	Human	+	N/D	+-	N/D	+
21PT ^c	Human	-	-	-	+	+
21NT ^c	Human	+	-	-	+	+
21MT-1 ^c	Human	+, Metastatic	-	-	+	+
TM3 ^d	Mouse	-	N/D	-	N/D	+
T-2410L TM6 ^d	Mouse	+	N/D	-	N/D	+
SP1 ^e	Mouse	+	+	+	+	+
SP1-3M ^e	Mouse	+, Metastatic	+	+	+	+

N/D: not determined

Legend:

- EL-E and WO-E are human breast carcinoma cell lines derived from human breast cancer patients (obtained from B. Campling, Cancer Research Labs, Queen's University).
- MCF10A1 is a subclone of a spontaneously immortalized non-tumorigenic human breast epithelial cell line established from long term culture of a breast subcutaneous mastectomy (22). MCF10A1neoT is a tumorigenic cell line derived following transfection of MCF10A1 with *Ha-Ras*. MCF10A1T3B is a cell line derived from a MCF10A1neoT tumor growing in a nude mouse (obtained from F. Miller, Michigan Cancer Foundation).
- Four breast cell lines were derived from a patient with infiltrating mammary carcinoma (23). 21PT is non-tumorigenic; 21NT is tumorigenic; 21MT-1 is metastatic.
- TM3 is a Balb/c mouse-derived mammary epithelial cell line (obtained from D. Medina, Baylor College). T-2410L TM6 is a malignant breast epithelial cell line derived from TM3.
- SP1 is a murine mammary carcinoma which arose spontaneously in a CBA female mouse. SP1-3M is a highly metastatic variant subclone of SP1 selected by serial passage of a lung metastatic nodule into the mammary fat pad (39).
- +- indicates trace amount detected.

FIGURE LEGENDS:

Figure 1: *In situ* hybridization (ISH) analysis of HGF and Met mRNA in Moderately (A-C) and well (D) differentiated human mammary infiltrating ductal carcinomas (IDC). ISH was carried out as described by Tuck *et al.* (11).

Panel A) ISH for Met mRNA showing intense cytoplasmic positivity at the advancing margin of the tumor (original magnification, x630).

Panel B) ISH for Met mRNA showed much weaker staining in the malignant epithelium in more central regions of the same tumor (original magnification, x250).

Panel C) ISH for HGF mRNA showing strong cytoplasmic positivity of the malignant epithelium at the advancing margin of the same tumor, in the same region which stained intensely for Met mRNA (original magnification, x630). In addition, stromal fibroblasts and epithelial cells showed some cytoplasmic positivity.

Panel D) ISH analysis for Met mRNA showing IDC surrounding a benign duct. Intensity of cytoplasmic staining is similar in both benign and malignant epithelium, regardless of whether the malignant epithelium forms well defined ductular structures (original magnification, x400).

Figure 2: Design of primers for reverse transcriptase polymerase chain reaction (RT-PCR) analysis of HGF, Met, MSP and RON mRNA: Design of primers and example of RT-PCR products obtained are shown. Details are provided in the Figure.

Figure 3: Cycle control of RT-PCR. To assess the linearity of PCR reaction in the quantitative RT-PCR technique for measuring mRNA, plasmids containing either human hepatocyte growth factor (*hHGF*) gene, pRS24, or human HGF receptor gene (*MET*), pMMET, and cDNA from a cell line, SP1, expressing both HGF and Met, were used in PCR reactions with ^{32}P end-labelled oligonucleotide.

Panel A) pRS24 plasmid DNA (50 ng) was used in each 10 μl reaction, which also contained 20 mM Tris (pH 8.3), 50 mM KCl, 0.2 mM dNTPs, 1.5 mM MgCl_2 , 0.1 μM of end-labelled oligonucleotide 1594, 0.9 μM unlabelled oligonucleotide 1594, 1 μM oligonucleotide 1595 and 1 U of Taq DNA polymerase (Gibco/BRL). Samples were then incubated at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and removed from the PCR machine after the indicated number of cycles. The PCR reactions were then resolved on 1.5 % agarose gel at 75 V for 2 hours. Bands corresponding to HGF PCR products were excised and counted by liquid scintillation.

Panel B) shows similar analysis of *HGF* amplification as in Panel A, but using cDNA generated from RNA isolated from SP1 cells.

Panels C and D) are similar analyses of *MET* gene using plasmid pMMET and cDNA from SP1 cells, and the primer pair oligonucleotide 1552 and oligonucleotide 1553. Linearity of the PCR reaction is observed between cycles 20 and 28 in all panels.

Figure 4: Examples of RT-PCR analysis of HGF and Met mRNA in mammary and non-small cell lung carcinomas.

Panel A) The mouse mammary carcinoma cell line SP1 (lane 1), human breast carcinoma cell lines WO-E (lane 3) and EL-E (lane 4), and human non-small cell lung carcinoma cell line SW-900 (lane 2) were tested by RT-PCR for Met mRNA expression. First strand cDNA were prepared by exposing 1 μ g of total RNA with a random primer using the First-Strand cDNA Synthesis Kit from Pharmacia Biotech. One tenth of the mixture from the reverse transcriptase reaction was subjected to RT-PCR for 25 cycles consisting of 1 min at 95°C (denaturing), 1 min at 55°C (annealing), and 1 min at 72°C (elongation). All four cell lines showed detectable RT-PCR products for Met.

Panel B) The murine carcinoma cell line SP1 (lane 2), and non-small cell lung carcinoma cell lines, BH-E (lane 3) and SK-LC6 (lane 4), were tested by RT-PCR for Met mRNA expression. RT-PCR was carried out as in Figure 4A. Molecular weight markers are shown on the left. SP1 and SK-LC6 cells but not BH-E cells showed detectable RT-PCR products for Met.

Panel C) The human breast carcinoma cell line WO-E (lane 3) was tested by RT-PCR for HGF mRNA expression. Plasmid containing human HGF gene, pRS24, was used as a positive control in lane 2. RT-PCR was carried out as in Figure 4A. Molecular weight markers are shown on the left. WO-E cells showed no detectable RT-PCR product for HGF.

Panel D) The non-small cell lung carcinoma cell lines FR-E (lane 2) and SK-LC6 (lane 3) were tested by RT-PCR for HGF expression. RT-PCR was carried out as in Figure 4A. Molecular weight markers are shown on the left. SK-LC6 but not FR-E cells showed a detectable RT-PCR product for HGF.

Figure 5: Purification of HGF by copper (II)-immobilized affinity chromatography from breast and non-small cell lung carcinoma cell lines.

Panel A) CM from three human mammary carcinomas, EL-E, MCF10AT3B and WO-E, were tested for HGF using copper(II)-immobilized affinity purification. Eluates were concentrated and resolved by SDS-PAGE under non-reducing conditions, followed by western blotting with sheep anti-HGF antibody, and secondary horse-radish peroxidase-conjugated anti-goat antibody. Membranes were exposed using chemiluminescence (ECL). EL-E and MCF10AT3B, but not WO-E, showed immunoreactive HGF protein.

Panel B) Conditioned medium (CM) from HEL299 cells (human embryonic lung fibroblast cell line) was concentrated from 30 ml to 5 ml, and subjected to copper (II)-immobilized affinity chromatography (69). The eluate from the column was further concentrated and subjected to 10% SDS-PAGE under non-reducing conditions. A positive control of rHGF (100 ng) was also included. Western blotting was performed as in Figure 5A.

Panel C) Copper (II)-immobilized affinity chromatography for purification of HGF from CM is demonstrated using CM from a non-small cell lung carcinoma (NSCLC) cell line, BH-E, and HEL299 cells, as indicated. rHGF (100 ng) and copper (II)-immobilized affinity chromatography purified rHGF are shown as positive controls. The eluates were subjected to 10% SDS-PAGE under non-reduced conditions, followed by western blotting as in Figure 5A.

Panel D) CM from three NSCLC cell lines were tested for HGF using copper (II)-immobilized affinity purification (69). The eluates were subjected to 10% SDS-PAGE under non-reducing conditions, followed by western blotting as in Figure 5A. CM from LC-T cells and QU-DB cells

are shown to be negative for detectable levels of HGF, compared to CM from BH-E cells, which demonstrated a detectable level of HGF.

Figure 6: Assessment of HGF activity in CM from HEL 299 fibroblast and BH-E carcinoma cell lines. CMs from HEL 299 fibroblast and BH-E carcinoma cell lines were purified by copper (II)-immobilized affinity chromatography, and subjected to 10% SDS-PAGE under reducing (2.5% β -mercaptoethanol v/v), and non-reducing conditions as indicated, followed by western blotting with polyclonal anti-human HGF antibody as in Figure 5A. rHGF (100 ng) was used as a positive control. The lower M_r band at 65-75 kDa under reducing conditions represents the dissociated α chain of HGF.

Figure 7: Sequence of Met used to develop antibodies. A fragment from the carboxy-terminus of human Met cDNA (Val₁₂₉₈ - Ser₁₃₉₄) was inserted into the pGEX-His vector (kindly provided by Dr. Peter Greer, Queen's University) using specific oligonucleotides (# 965 and #966, as indicated). The oligonucleotides contained a BamHI and EcoRI restriction site to allow for insertion into the plasmid. The plasmid contained ampicillin resistance (amp^r), lac repressor (*lacI*^q), lac promoter (P_{lac}), a six histidine tag (HisHisHisHisHis), and glutathione S-transferase sequence (GST).

Figure 8: Detection of Met in human breast and non-small cell lung carcinoma cell lines.

Panel A) The MCF10A series of breast cell lines were tested for Met expression. Whole cell lysates from MCF10A1, MCF10A1neoT, and MCF10AT3B were adjusted to equal protein concentrations, immunoprecipitated with MET1 antibody, and subjected to 10% SDS-PAGE under reducing conditions (2.5% β -mercaptoethanol, v/v). Proteins were then transferred onto a nitrocellulose membrane, and western blotting was performed with anti-human Met antibody (UBI, clone DQ-13), followed by secondary horse-radish peroxidase-labelled anti-mouse antibody. The A549 cell lysate was used as a positive control (lanes 4 and 5). MET1 serum alone is shown as a negative control.

Panel B) EL-E, MCF10AT3B and WO-E breast carcinoma cell lines were tested for Met expression. Whole cell lysates were immunoprecipitated with MET1 antibody and subjected to 10% SDS-PAGE under reducing conditions, followed by western blotting with anti-human Met antibody, as described in Figure 8A.

Panel C) Eight non-small cell lung cancer cell lines were tested for Met expression by immunoprecipitation with MET1 antibody. Immunoprecipitates were subjected to 10% SDS-PAGE under reducing conditions, followed by western blotting with anti-human Met antibody as in Figure 8A. The A549 cell lysate was used as a positive control (lane 9).

Figure 9: Assay for activation of Met in A549 cells. A549 cells were serum-starved overnight and incubated without, or with, HGF (40 ng/ml) for 30 min. Cells were lysed, and equal amounts of protein immunoprecipitated with anti-Met antibody (MET1) or anti-phosphotyrosine (PY) (UBI, PY20) antibody, as indicated. Immunoprecipitates were subjected to 10% SDS-PAGE under reduced conditions. Proteins were transferred to nitrocellulose membranes, and blotted with anti-Met antibody (clone DQ-13) (Panel A), or anti-PY antibody

(Panel B). The results show HGF-dependent tyrosine phosphorylation of Met in A549 cells.

Figure 10: Detection of Met activity in nonmalignant and transformed breast epithelial cell lines.

Panel A) MCF10A1 and MCF10A1neoT (transfected with *Ha-Ras* cDNA) cells were incubated for 30 min without, or with, monoclonal Met-activating antibody (clone #6G1, Genentech). Cells were lysed, and the proteins were immunoprecipitated with anti-phosphotyrosine antibody (PY20). Immunoprecipitates were subjected to 10% SDS-PAGE, under reducing conditions, followed by western blotting with anti-human Met antibody (Clone DQ-13) as in Figure 8.

Panel B) MCF10AT3B cells were incubated with serum-free medium, conditioned medium from 3T3-L1 adipocytes (which contains HGF (69)), or monoclonal Met-activating antibody (clone # 6G1), lysed, and the proteins were immunoprecipitated with anti-phosphotyrosine antibody (PY20). Immunoprecipitates were subjected to 10% SDS-PAGE under reducing conditions, followed by western blotting with monoclonal anti-human Met antibody as in Figure 8.

Panel C) CMs from EL-E and MCF10A1T3B cells, or rHGF (40 ng/ml) were incubated with A549 cells for 30 min. A control consisted of A549 cells incubated in medium alone. Cells were then lysed, immunoprecipitated with anti-MET1 antibody. Immunoprecipitates were subjected to 10% SDS-PAGE under reducing conditions, followed by western blotting with anti-phosphotyrosine antibody (PY20). Reprobing with anti-Met Antibody (clone DQ-13) indicated equal amounts of Met protein in each track (data not shown).

Figure 11: Promoter structure of murine hepatocyte growth factor gene. The 5'-unsaturated sequence and exon of murine HGF gene is shown. The transcriptional start site (+1) and the TATA element are indicated. Potential transcription factor binding sites are shown with arrows indicating the orientation of these elements. This figure is derived from Liu *et al.* (34). Additional transcription factor binding sites were found searching the sequence with TFSEARCH version 1.3 program with a minimum of 85% identity of each binding site to the consensus. See text for details.

Figure 12: The effects of different inhibitors on SP1 proliferation. SP1 cells were starved overnight in serum-free medium before being harvested for proliferation assays. Cells (10^4 per well) were seeded in 1 ml of medium containing the indicated amount of FBS or drug. The inhibitors used were as follows: Panel A, farnesyl-transferase inhibitor B956; Panel B, tyrosine kinase inhibitor, herbimycin A; Panel C, PI 3-kinase inhibitor, LY294002; and Panel D, transforming growth factor- β (TGF- β). Each group was done in quadruplicate. In all assays, cells were grown in RPMI 1640 medium except for Panel D, where cells were grown in DMEM medium. Twenty four hours after seeding, 0.2 μ Ci of [3 H]-thymidine were added to each well, and incubated for an additional 24 h at 37°C with 5% CO₂. Cells were then harvested using trypsin-EDTA and transferred onto filter paper using a Titerek cell harvester. Incorporation was determined by liquid scintillation counting.

Figure 13: Glutathione S-transferase fusions used in HGF protease detection. Schematic diagrams of GST-HGF fusions are shown with their expected molecular weight (MW), and these proteins are A) glutathione S-transferase (GST), B) GST-HGF(aa 485-544) with the cleavage site for HGF processing at Arg₄₉₅-Val₄₉₆(marked by a star), C) GST-HGF fusion expected when Arg₄₉₅

site is cleaved by HGF specific protease. To construct the GST-HGF (aa 485-544) fusion, a portion of *HGF* cDNA encoding amino acids 485-544 was generated by polymerase chain reaction using oligonucleotides #1027 and 1028, followed by ligation of the PCR product into BamHI/EcoRI sites of pGEX1 plasmid, which harbours GST coding sequence preceded by lac promoter. The resulting plasmid, pGEX-HGF, was then transformed into *E. coli*. Expression of GST-HGF fusion from bacteria was induced by addition of 0.25 mM IPTG to the media and incubation at 25°C for 3 hours. Isolation of the GST fusion was achieved by using glutathione Sepharose 4B (Pharmacia).

Figure 14: Antisense oligonucleotides used in this study.

Panel A) Six antisense oligonucleotides containing C-5 propyne pyrimidine and phosphorothioate modifications, were designed with specificity for both human and murine *met* genes to downregulate expression of Met protein. The target site of each antisense oligonucleotide is shown in Figure 16. The corresponding mismatch oligonucleotides to be used as controls are also shown.

Panel B) Six antisense oligonucleotides containing the C-5 propyne pyrimidine and phosphorothioate modifications, were designed with specificity for both human and murine *HGF* genes to downregulate expression of HGF protein. The target site of each antisense oligonucleotide is shown in Figure 15.

Figure 15: Location of target sites of antisense oligonucleotides against *HGF*.

Panel A) The genomic organization of the *HGF* gene is shown.

Panel B) The position of the RT-PCR product for *HGF* is also shown.

Panel C) The target sites of six antisense oligonucleotides (ANS1 to ANS6) against *HGF* are shown. SP, signal peptide; HP, hairpin; ANS, antisense.

Figure 16: Location of target sites of antisense oligonucleotides against *Met*.

Panel A) The genomic organization of the *MET* gene is shown.

Panel B) The position of the RT-PCR product for *MET* is also shown.

Panel C) The target sites of six antisense oligonucleotides (ANS1 to ANS6) against *MET* are shown. SP, signal peptide; TM, transmembrane domain; ANS, antisense.

Figure 17: Photomicrograph of incorporation of FITC-labelled oligonucleotide into SP1 carcinoma cells. SP1 cells were grown in DMEM with 0.25% FBS on gelatin-coated glass coverslips in a 24-well dish. The cells were treated with FITC-labelled oligonucleotide (50 nM) and Cytofectin GS2888 (2 µg/ml) as carrier, in 1 ml of DMEM with 0.25% FBS at 37°C in a 5% CO₂ incubator. After 4 h, the cells were fixed with 2% paraformaldehyde in PBS for 30 min at room temperature. The coverslips were observed under an fluorescence microscope (original magnification, x480).

Figure 18: Effect of antisense to the *MET* gene on proliferation of SP1 carcinoma cells.

SP1 cells were plated at 10⁴ cell/well in 0.25% FBS DMEM alone (control), or with HGF (20 ng/ml), or with Cytofectin (GS2888, 2 µg/ml) alone, or with unrelated oligonucleotide (50 nM) or *MET* antisense oligonucleotide (ANS1) at concentrations indicated. DNA synthesis was measured by

adding [³H]-thymidine (0.2 μ Ci/well) at 24 h and incubating for an additional 24 h before cell harvest. Cells were harvested using trypsin/EDTA, transferred onto filter paper using a Titertek cell harvester, and counted in a scintillation counter. The results are expressed as the mean (CPM/well) +/- S.D. of triplicates determinations. *MET* antisense oligo #1 specially inhibited proliferation compared to the unrelated oligonucleotide and to the GS2888 cytofectin control.

Figure 19: Model of stromal-tumor interactions. This model summarizes the overall hypothesis of the role of HGF and extracellular matrix in breast cancer based on results in our laboratory. HGF, produced by stromal cells stimulates nonmalignant and early malignant cells in a paracrine manner. Aberrant expression of HGF by carcinoma cells and coexpression of HGF and HGF receptor provides a selective advantage for survival, invasion and metastasis. In addition, fibronectin can act co-operatively with HGF via $\alpha 5\beta 1$ integrins, to augment the survival and proliferation of carcinoma cells (Cell and Mol. Biol. 43:455-468, 1997). Stars indicate possible targets for therapeutic intervention. Thus, tissue microenvironment may be important in the regulation of progression of mammary carcinomas.

Figure 1

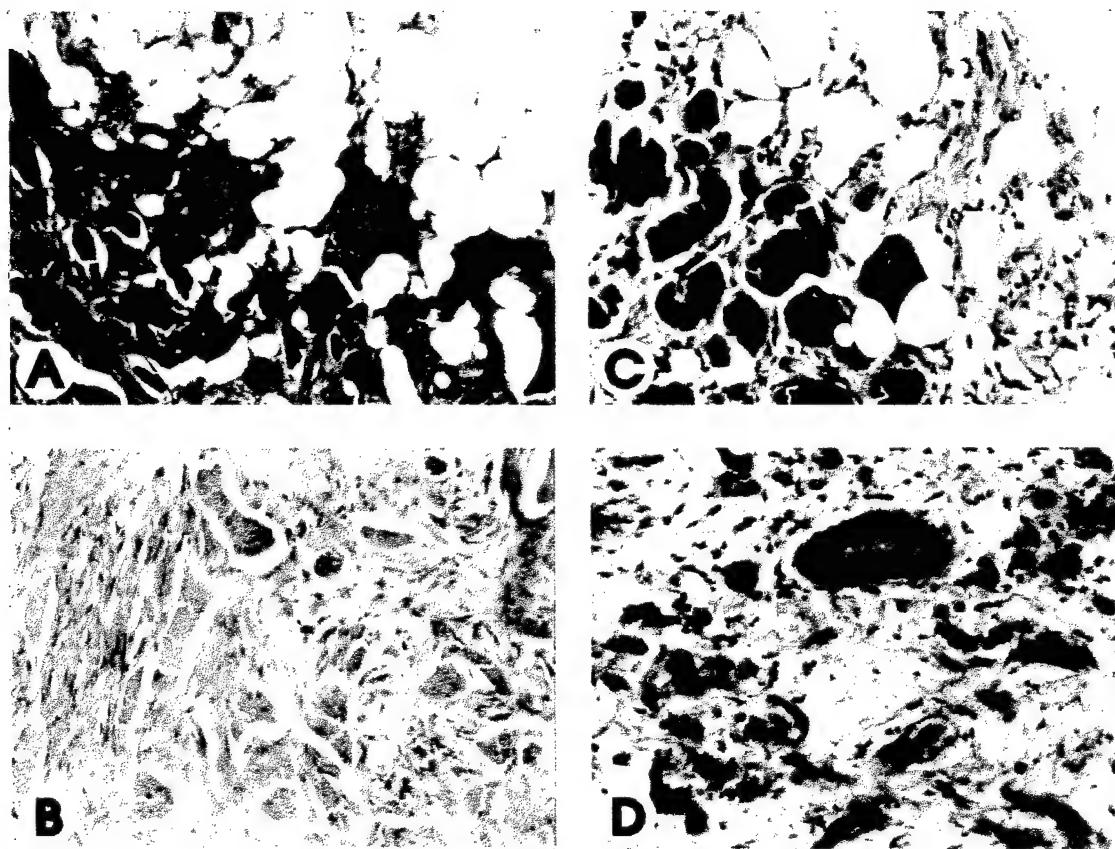


Figure 2

A

HGF and MSP RT-PCR primers (Human and Mouse):

- The primer for HGF:

5' primer is the sense sequence

5'	TGT CGC CAT CCC CTA TGC AG
Human(69-88):	CAT CGC CAT CCC CTA TGC AG
Mouse(72-91):	TGT CGC CAT CCC CTA TGC AG

3' primer is the antisense sequence

5'	TCA ACT TCT GAA CAC TGA GG
Human(610-629):	TCA ACT TCT GAA CAC TGA GG
Mouse(613-632):	TCA ACT TCT GAA CAC TGA GG

The PCR product will be 560 bp long

- The primer for MSP:

5' primer is the sense sequence

5'	CCA CTA CAA CAT GAG CAG CC
Human(207-226):	CCA CTA CAA CGT GAG CAG CC
Mouse(207-226):	CCA CTA CAA CAT GAG CAG CC

3' primer is the antisense sequence

5'	CTC CCT GCA GGT TTT GAT GC
Human(545-564):	CTC CCG GCA GGA TTT GAT GC
Mouse(545-564):	CTC CCT GCA GGT TTT GAT GC

The PCR product will be 357 bp long

Met and RON RT-PCR primers (Human and Mouse):

- The primer for MET:

5' primer is the sense sequence

5'	CAT GGC TCT GCT TGT TGA CAC
Human(351-371):	CAT GGC TCT AGT TGT CGA CAC
Mouse(351-371):	CAT GGC TCT GCT TGT TGA CAC

3' primer from is the antisense sequence

5'	GAC AGG AGG ACT TTG GCT CC
Human(541-560):	GAT ACC AGG ACT TTG GCT CC
Mouse(538-557):	GAC AGG AGG ACT TTG GCT CC

The PCR product will be 206 bp long

- The primer for RON:

5' primer is the sense sequence

5'	CCT TGA CTT CTT CCA GAC GCC CAG
Human(1191-1214):	CCT CGA CTT CTT CCA GTC GCC CAG
Mouse(1197-1220):	CCT TGA CTT CTT CCA GAC GCC CAG

3' primer from is the antisense sequence

5'	CCA CAC CAG CCA CAT CCC ATG AA
Human(1624-1646):	CCA CAC CAG CCA CAG CCC ATG AA
Mouse(1630-1652):	CCA CAC CAG CCA CAT CCC ATG AA

The PCR product will be 455 bp long

Legend

Figure 2: Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of HGF, Met, MSP and RON mRNA

Panel A: Design of PCR primers for detection of HGF, Met, MSP and RON mRNA.

The primers were designed to overlap more than one exon and will not cross react with each other. The final products are different sizes in order to detect which mRNA species is expressed.

Panel B: Detection of mouse Met and HGF mRNA by RT-PCR.

cDNA was prepared by exposing 1 μ g of total RNA with random primer to reverse transcriptase. The cDNA obtained was subjected to RT-PCR of 25 cycles consisting of 1 min at 95°C (denaturing), 1 min at 55°C (annealing), and 1 min at 72°C (elongation). DNA molecular weight markers are in lane 1. The PCR product of Met (lane 2) can be detected as a 206 bp band. The PCR product of HGF (lane 3) can be detected as a 560 bp band.

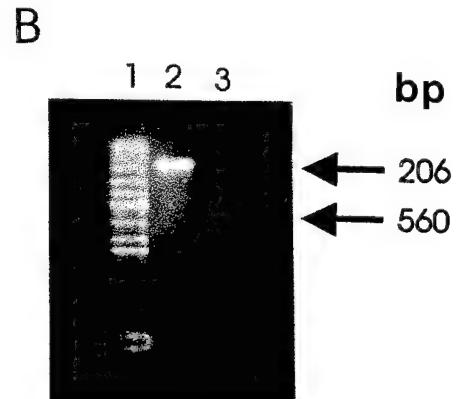


Figure 3

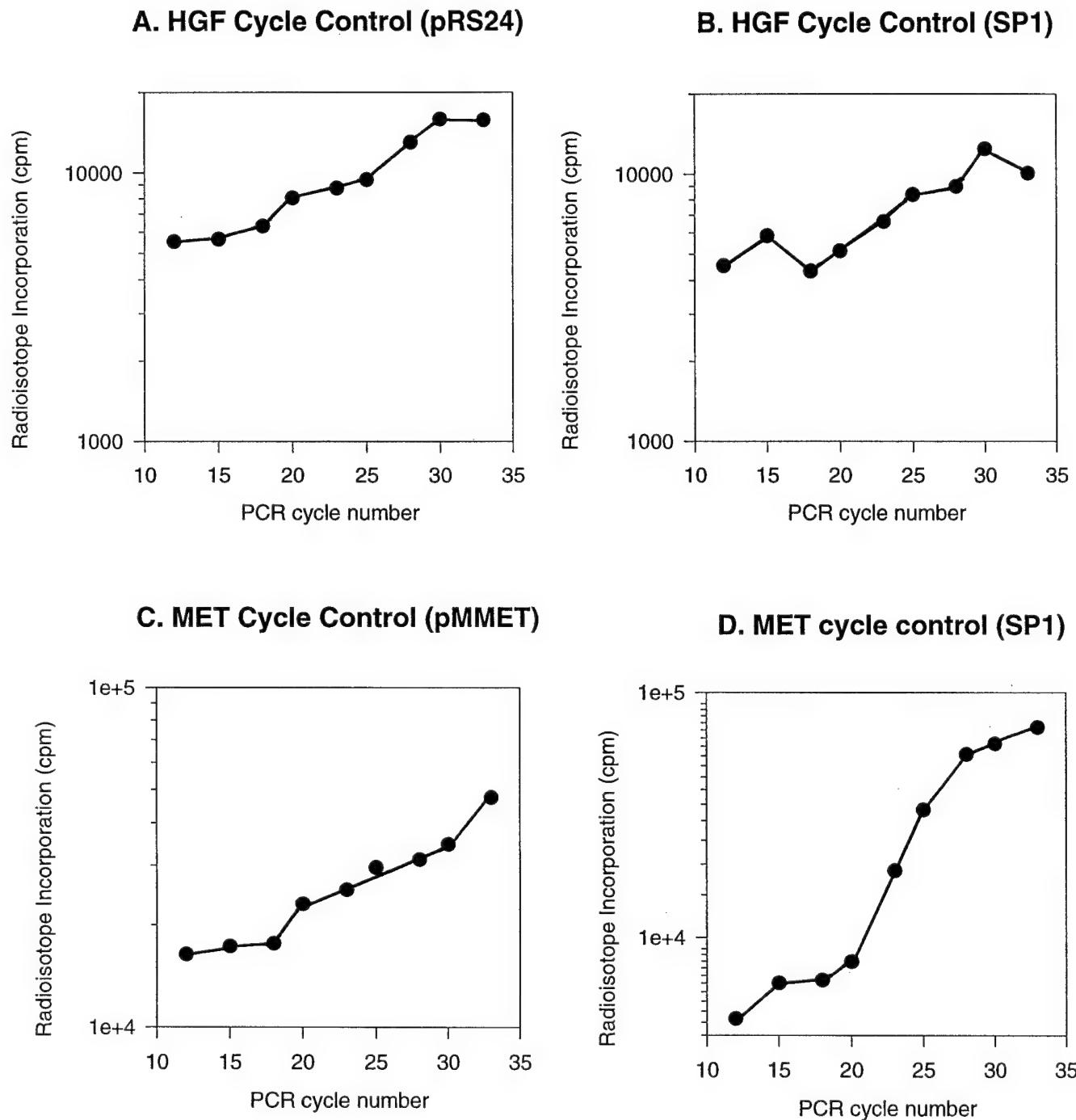
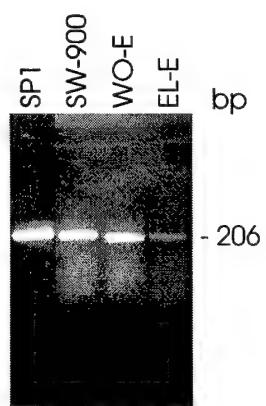


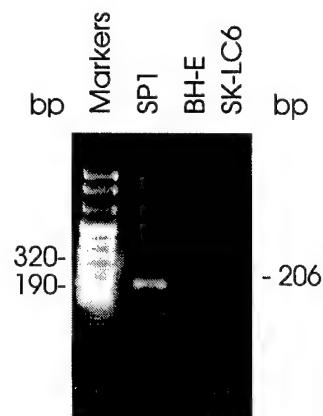
Figure 4

RT-PCR Detecting MET

A

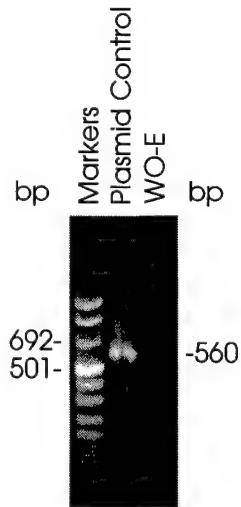


B



RT-PCR Detecting HGF

C



D

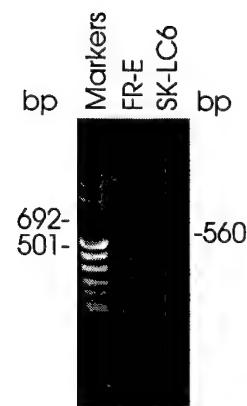


Figure 5

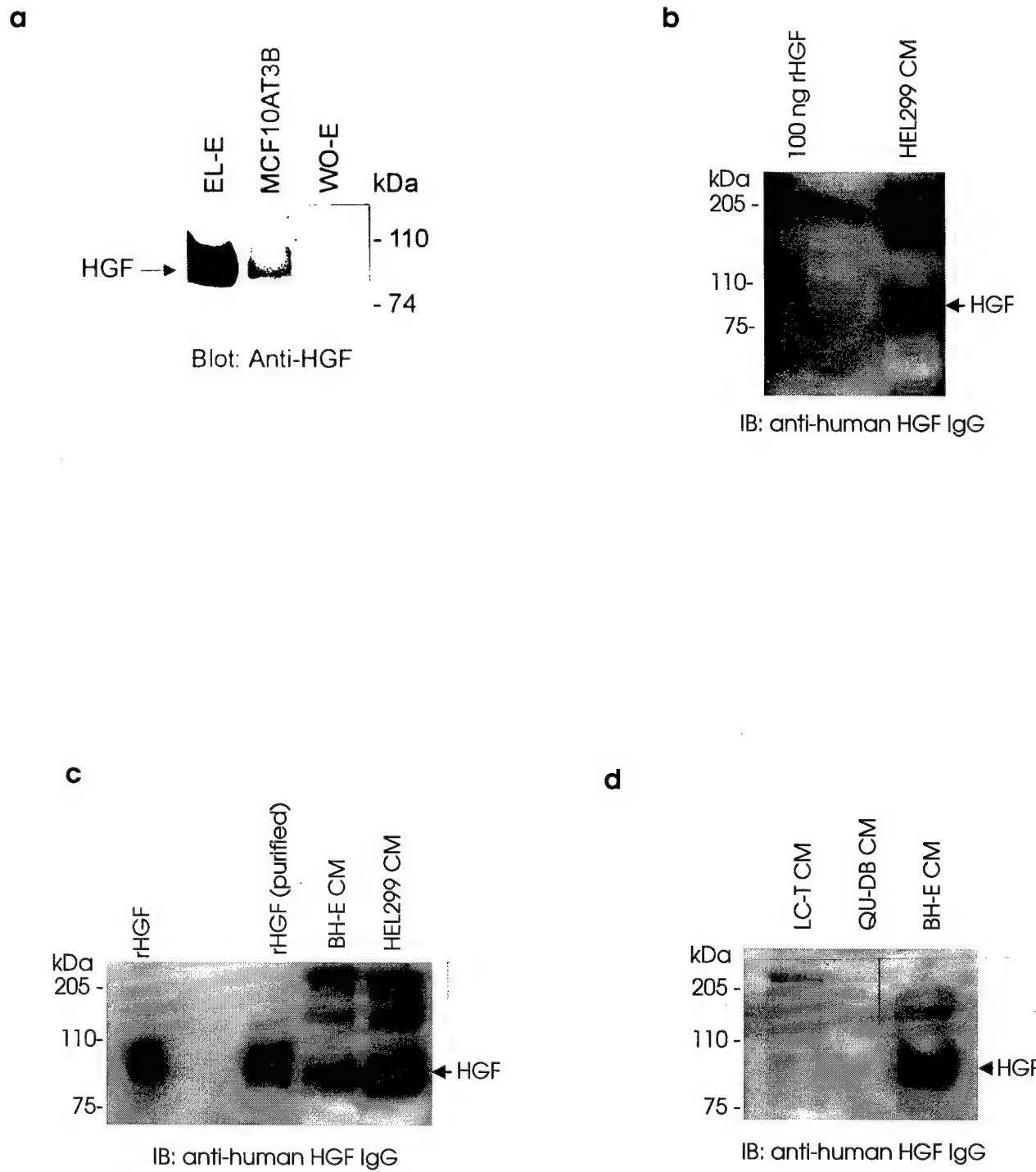


Figure 6

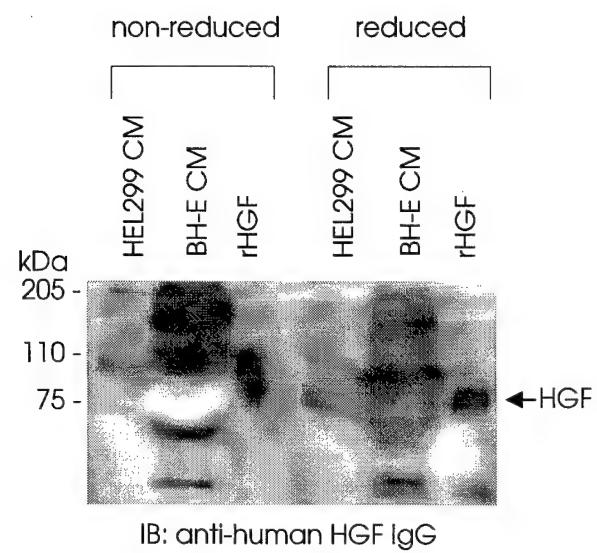
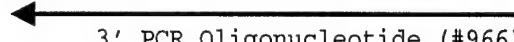
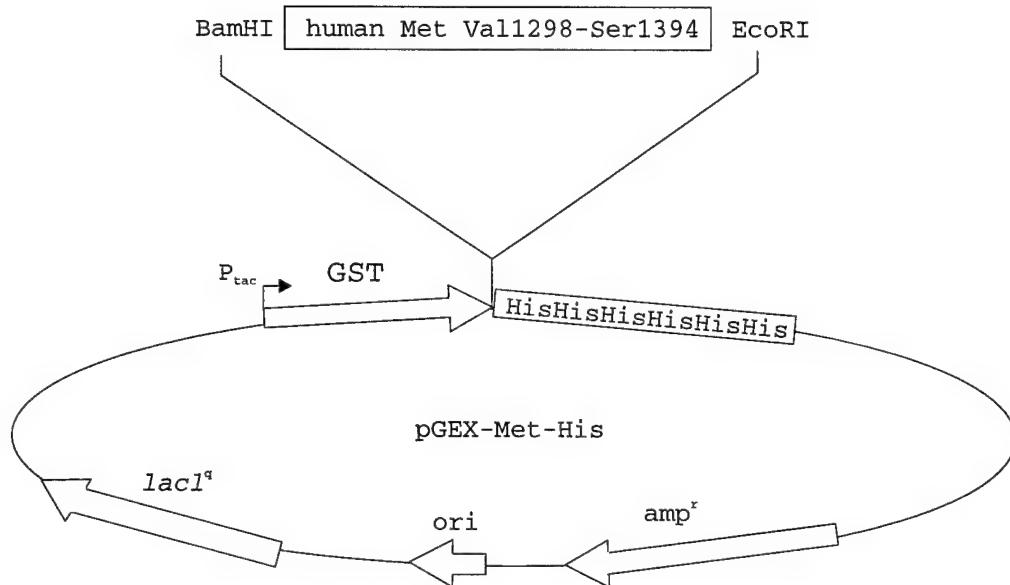


Figure 7

5' PCR Oligonucleotide (#965) 

Asp Val Tyr Leu Leu Glu Gly Ser Phe Trp Glu Thr Ser ***
 5' - GAT **GGA TCC** GTT TAC TTG TTG CAA GGG... TCC TTC TGG GAG ACA TCA TAG TGC TAG T-3'
 Bam HI 3' - GG AAG ACC CTC TGT AGT **ACT TAA GTC** A-5'
 EcoRI

3' PCR Oligonucleotide (#966) 



pGEX-His

GST: Thrombin cleavage site

Pro Lys Ser Asp Leu Val Pro Arg Gly Ser Pro Gly Ile His His His His His His
 CCA AAA TCG GAT CTG GTT CCG CGT **GGA TCC** CCG **GAA ATT CAT** CAC CAT CAC CAT CAC
 BamHI 
 SmaI

Figure 8

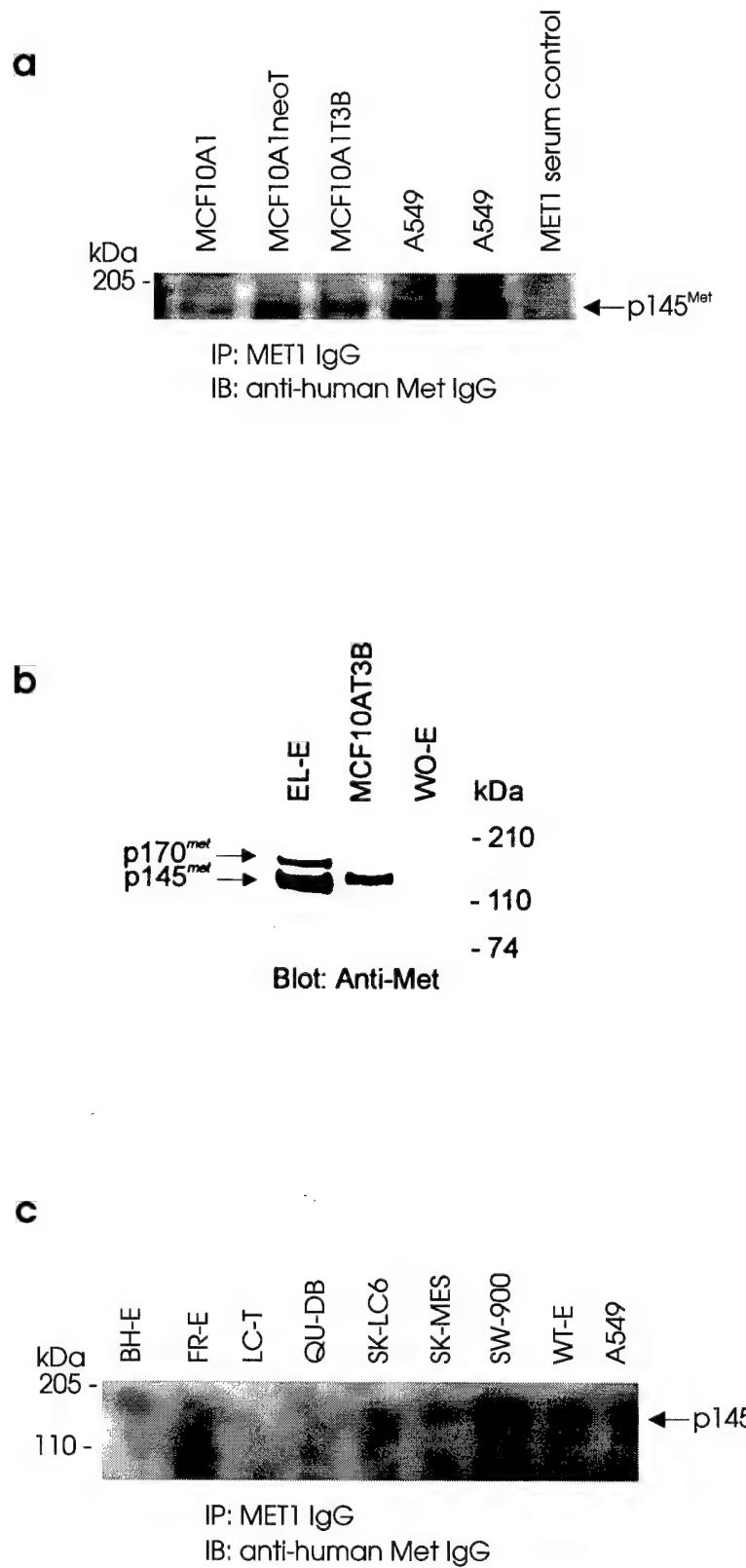
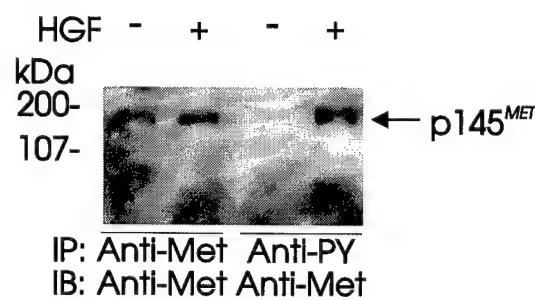


Figure 9

A



B

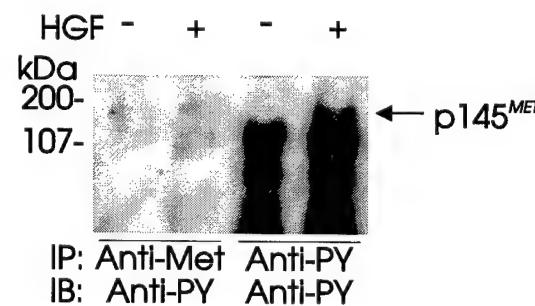


Figure 10

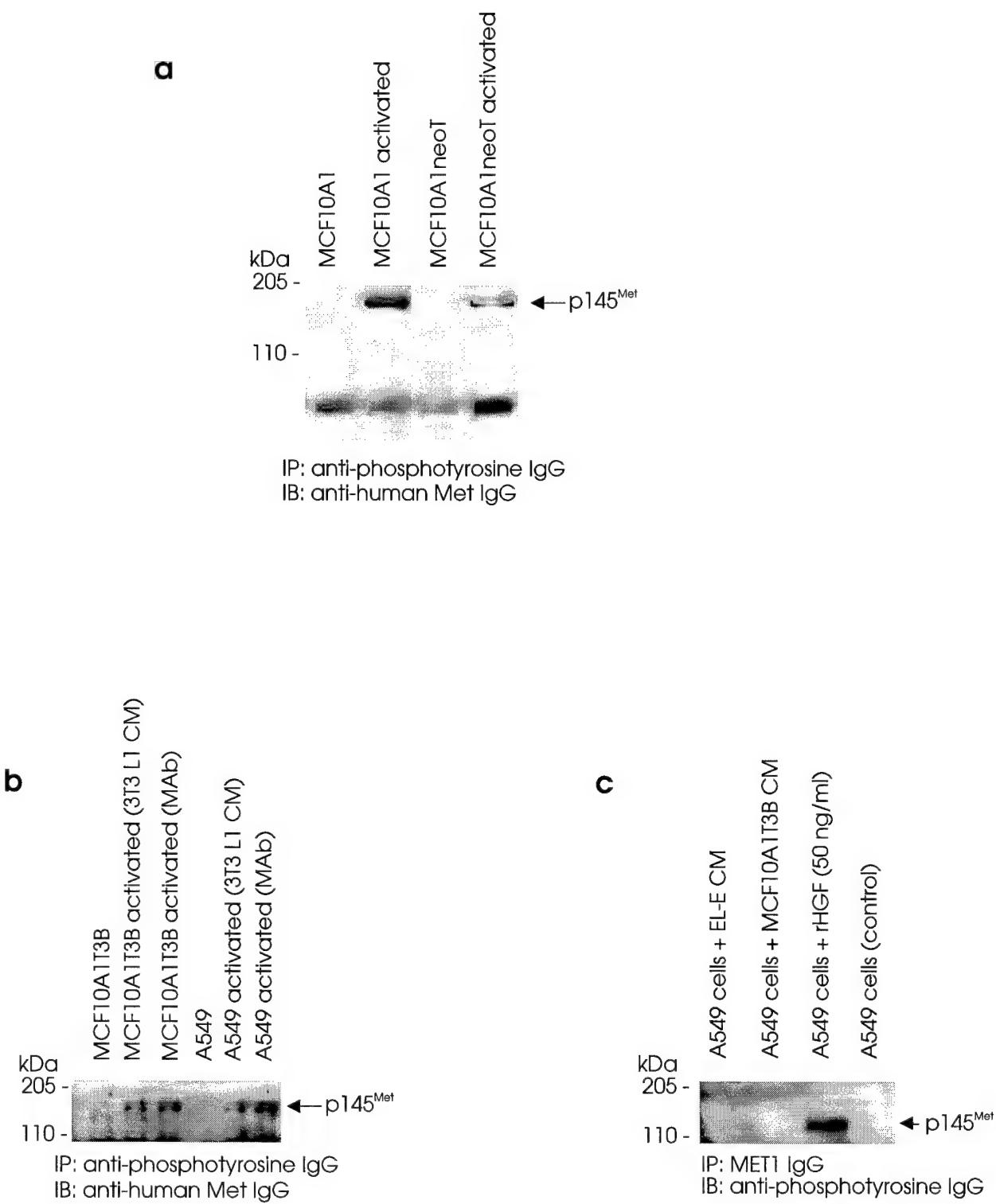


Figure 11: Promoter Structure of Murine Hepatocyte Growth Factor (HGF) gene.

-2845 gtcgactcga tcatgaaatc ctagctaccc atgaggccaa aataaagtta ttttcctat
 -2785 agattatggt cgatctactt gaaactaaca cctgagtatt tactactttg tcaccttgaa
 -2725 caattcacct gaatataaac agctttctta agcttcgttg actatgataat ggagactata
 -2665 tggaccgtct tatgtttatc ccaagggtta tataacaaca tatgaggact taaaacgttt
HIF1 ← **Ets** →
 -2605 agcatgtact aaaagatcaa tgaacattag ctaagattc aatgaagagg aaccaatacc
 -2545 ttgtcagaat aaaatgaaag gaataggtaa tttaaaatag atgtgtgttt actgtactta
 -2485 otgacaattha aatttatgaa aatatacatg ttgctctgtc taatttcaag ttatthaagta
 -2425 ctaatctttt aaaattgatt ttatttagtag atattaatta tatattattt ttttcattat
 -2365 gacatttga catagtatat tctgatcaaa ttactgcca aattatcatt ttctgtccc
IL-6 RE ← **AP1** →
 -2305 ttcccttc tcactttca ccttcttctt cccagagagt catccttcta cctctcaagg
 -2245 ttgtttgt ttgtttgt ttgttttc tcttttgaa gtatgggtg acctaattgag
 -2185 catcattagc atgggtcaca agaggagtgt gggcaaccac agaagaaaat gtaattcatt
 -2125 cagcaactat taactggcca tagactgaca agaccctgag ggagggcttt gtccctcatga
 -2065 gagaagagac aaacagaaac acagatcaca tcagttccat tcattctacc tacacaattt
 -2005 acggggaaat tgcttcctaa aattccctt tactttccaa caagctcta aatatgttat
GATA →
 -1945 agggcaactc agagttgtgg tcagcagcat attgaaatgg agatgttttta gcaaagggtcc
GATA →
 -1885 atgcactcat taaaaagtaa actagataat aggaaaatgg gcgggaacct tttttttta
 -1825 aagcgtgtt ggattagctt tgtatgcaaa agtggtgaaa gatagagcaa tggaaacag
GATA ←
 -1765 ttaatagaaa gatatccttc agcactgatc gatcttccaa tgttcctggg ttcaaggaca
GATA ←
 -1705 accatctgca actccagtct gtaggcaata ggcattgcaag tggtatacag acataaatgc
HIF1 ←
 -1645 atgcaaaaca ctcatacaca taaaactaaa aatcctgaa agaaattctc cttcattact
GATA ←
 -1585 agattctcca tagtcccaag gacttatcaa cctagcttct ttctttctt cttctccct
 -1525 cccttcatt ttcttcctc ttcttcttc ctacctctct ttttccctt cttccctt
STAT ← **COUP** →
 -1465 ttctgcctt cccttctct ttctccctt ccctgaactt tccccttctt cttgtctctg
 -1405 caggtggctg tagctatgct ggaggagggt gaagagatct gccttgctg gtggaggtgg
 -1345 agaaaggcca ccctattact gactttcatc ttgccttgg gtacctctgt atggcagcag
 -1285 gcaactctcc agtagacaat gtctcacatt ctgtcgactt tcccttctc taaagaggta
GATA ←
 -1225 tttagttcctt tgggtatcta gtctcgagaa atacagaggt atctatgcct caaaatctat
SSRE →
 -1165 tagagacaca gtacgctatc tctgacacat cagagcaccc accaggtgtt gtaataaaaag

Figure 11: Promoter Structure of Murine Hepatocyte Growth Factor (HGF) gene. [continued]

HIF1 → GATA

-1105 gagaagtctg gacaatgatc tcacccaaac gtgatactca tggatttgag caccggctgc

-1045 ttcctccctc cctgaagact gtgtgatgct gtgggtgtgt gtaggagaaa agaggtaag

CRE ← SSRE

-985 acgtcattta ggaattaaaa tagtccaacg ggtctcaagt gaaagtctta gtgcatacat

CRE ← ERE →

-925 actgacgtgt gaacatgtgt aactgtacac aaggtcagaa agaccattat ctatttgctt

-865 gtcagggaga tgcaaagtgc catgtctatc ccttaagaga aattatcaac acacacatca

-825 gaaaacagac atttgaaca tttatggg atcctttgc agtggttgg ggtagcttt

-765 ctgagggaaag ctgccatgtc gagccacta gactggcaaa tttctggcc tgaaaaccag

GRE

-705 ggtgttttg tttctgggtgc tatttcttct cattcgtcaa ggccccacgc ttttcttacc

-645 tgccctcttt ctacctgggc tctgcacccc tgacttcctt actctgctct tactgtctt

STAT ← COUP/ERE →

-585 aaaatcccg gaaactgggg tcagtggtca tccctgaatc tctccagcac tgcaagctcg

-525 cagactatga gctggggctc atttggcata agggctgtcc gctctttct tatgctgctt

IL-6 RE →

-465 ccccttcctc tttcccaaga tagatatgtt aacacatgca tttctgttt caaacggggc

TIE ← AP1 →

-405 gaattgggt tctgcctgtc cttgactta gcgattgggc tgagccttgc tcctcccttc

GATA ← GATA →

-365 cctactcgga taggagccac aaggatctgg agctccagct tccaaattgc agctggcctc

IL-6 RE ← SSRE →

-305 gggccaggtg acctttgctt tgtaagttt tttctgggag ggggagtgtg tggagggggag

IL-6 RE ←

-245 agctggatc ttttgcgtt tggggggggg ggggggtttag gaaaggaagg ggcttggaaaga

-185 gagtaaaggg ctgttgttaa acagtttctt accgtaagag ggagttcaga cctagatctt

TATA

-125 tccagtaat cacacaacaa acttagctca tcgcaataaa agcagctcag aaccgaccgg

+1

-65 tttgcacag gattcttca gcccggcata tcctgcagag ggatcagtct gctgaaactg

Exon 1

-5 caagc **ATGAT GTGGGGGACC AAACCTCTGC CGGTCTGTGTT GCTGCTGCAT GTCCTCCTGC**

Exon 1

+57 **ACCTCCTCCT GCTTCATGTC GCCATCCCC ATGCAG** gtaa gtttacttct gttcatgg

+117 gattggggtt gcttaagtc tccttctgac cttactctt cttccaaga agctctctgc

+177 cctatttcca gcatccttcc ttaattccat gtgtgggtgt ggcttcaaaa cctctcatta

+237 tgctttcttt tgacattcct tttctgtttt gaaaacattt aaaataatta aatctctca

+297 gtgataacccc caccccccac caggccccac ctcatttagc caaacagtcc aaaattatgg

+357 gataggaatg aagtgctaac actggctgga actttgctgg tttggccaag tttaaaaacg

+417 gagacagatt ccgaattgaa gagaggagag tgatggtaa tctaaccagg gactcatgat

Figure 12

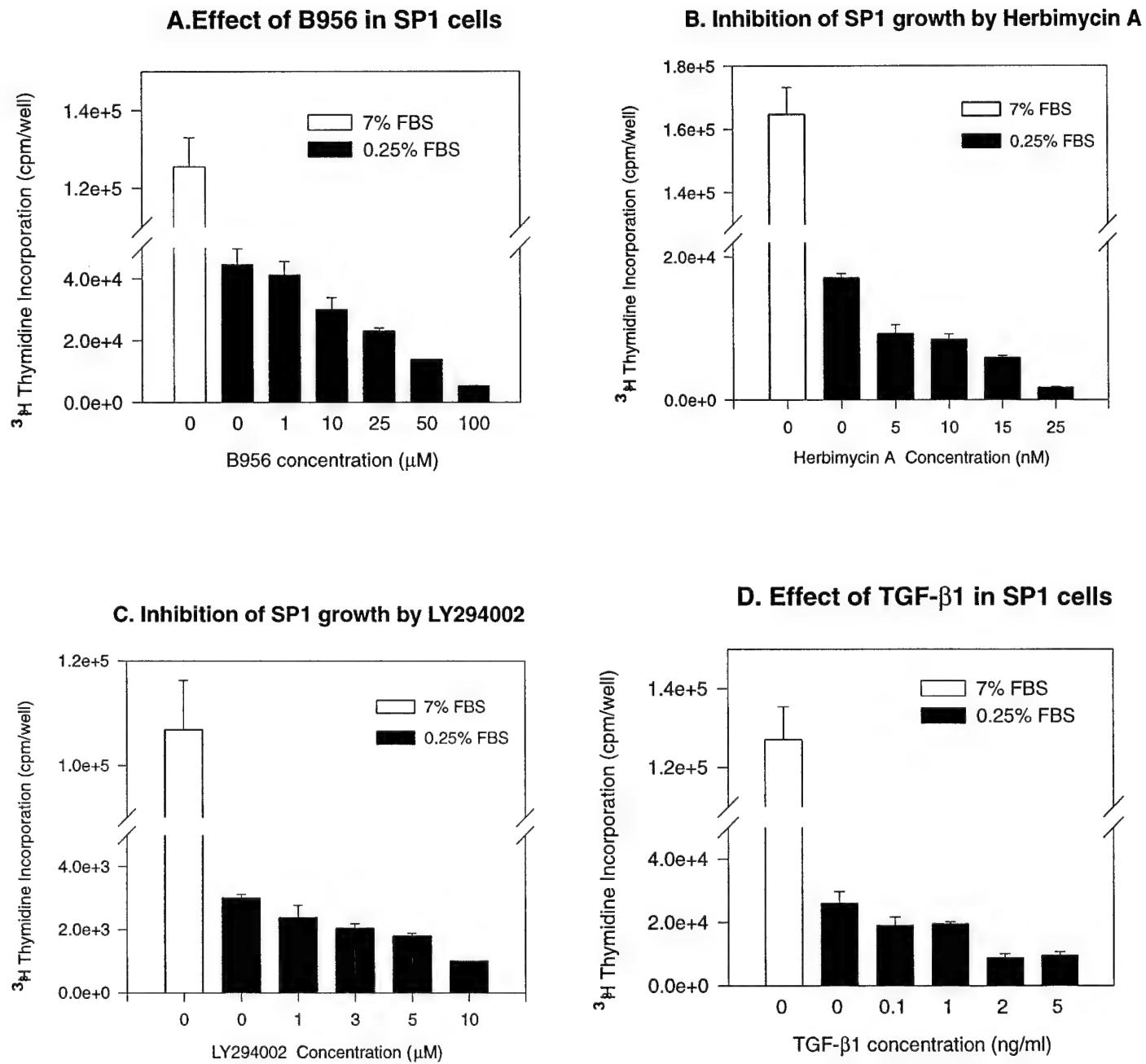


Figure 13

Glutathione S-transferase fusion proteins

A



MW (kDa)

27

B

Arg₄₉₅-Val₄₉₆



33

HGF
(aa485-544)

C



28.3

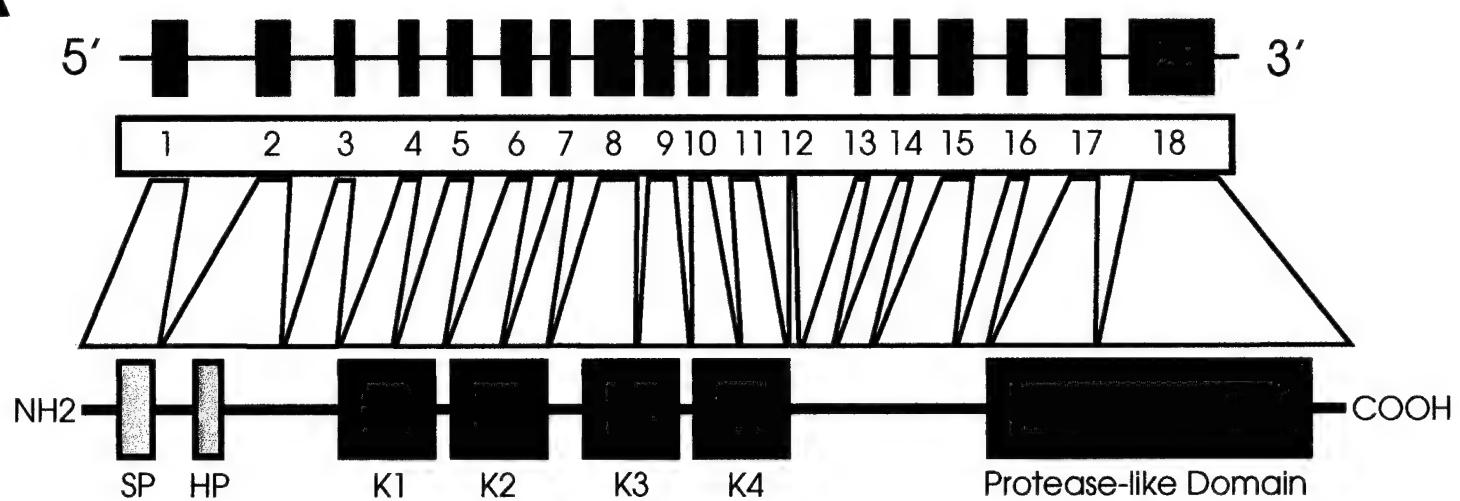
HGF
(aa485-495)

Figure 14

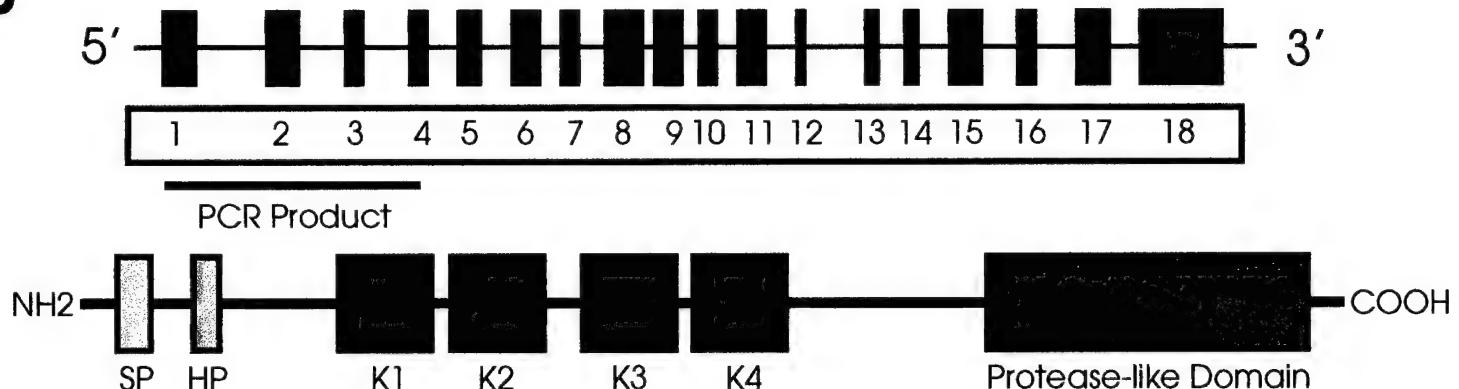
	Site (nt) (Mouse)	Sequence
Panel A: Met		
Antisense:	303-317	5' UUG GCU UUG CUG CUG 3'
Mismatch:		5' UUG GCG GUU CUU CUG 3'
Antisense:	343-357	5' AGC CAU GUU GAU GUU 3'
Mismatch:		5' AGC CAG GAU UUU GUU 3'
Antisense:	561-575	5' AUG AAC CGG UCC UCC 3'
Mismatch:		5' AUG ACC GGC UAC UCC 3'
Antisense:	1036-1050	5' UGG CUU GCU UUG UGC 3'
Mismatch:		5' UGG CUG UCU GUU UGC 3'
Antisense:	1145-1159	5' GCU GGU GAC AUC UCA 3'
Mismatch:		5' GCU GAU CAG GUC UCA 3'
Antisense:	1219-1233	5' GCG CGC UUC ACA GCC 3'
Mismatch:		5' GCG CAC AUC UCG GCC 3'
Panel B: HGF		
Antisense	39-53	5' AGG AGG ACA UGC UGC 3'
Antisense	102-116	5' GUA UUU CUU CUU UUC 3'
Antisense	313-327	5' CAC UCC ACU UGA CAU 3'
Antisense	357-371	5' UCU UUG UUU UCA UAG 3'
Antisense	516-530	5' UAG UUU UCC UGU AGG 3'
Antisense	591-605	5' CAG ACU UCG UAG CGU 3'

Figure 15

A



B



C

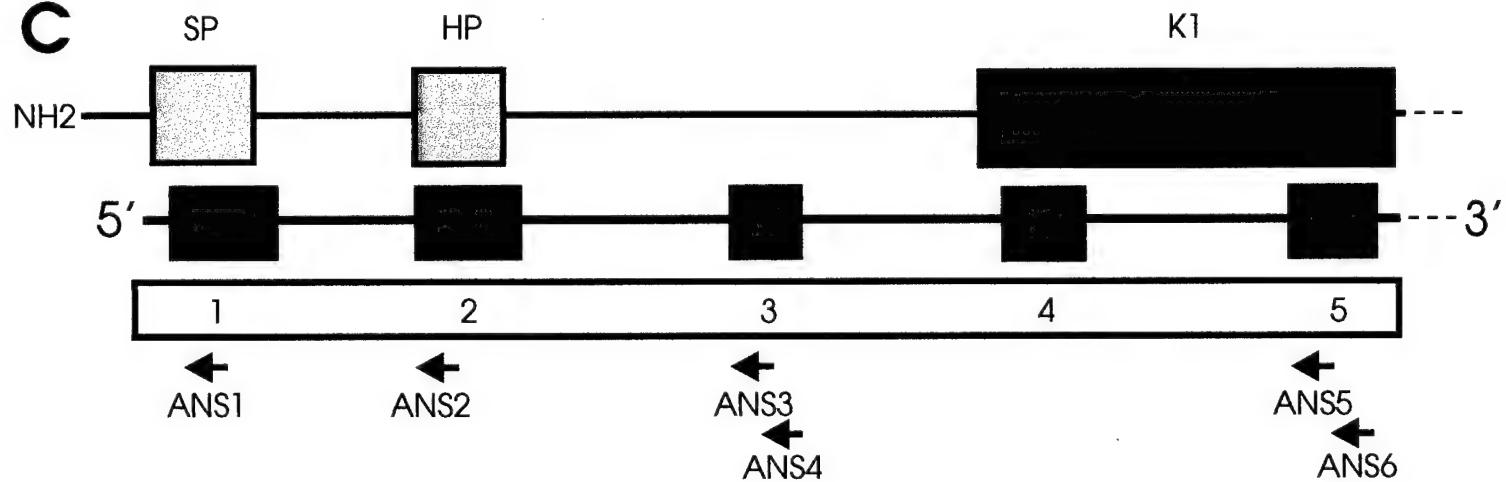
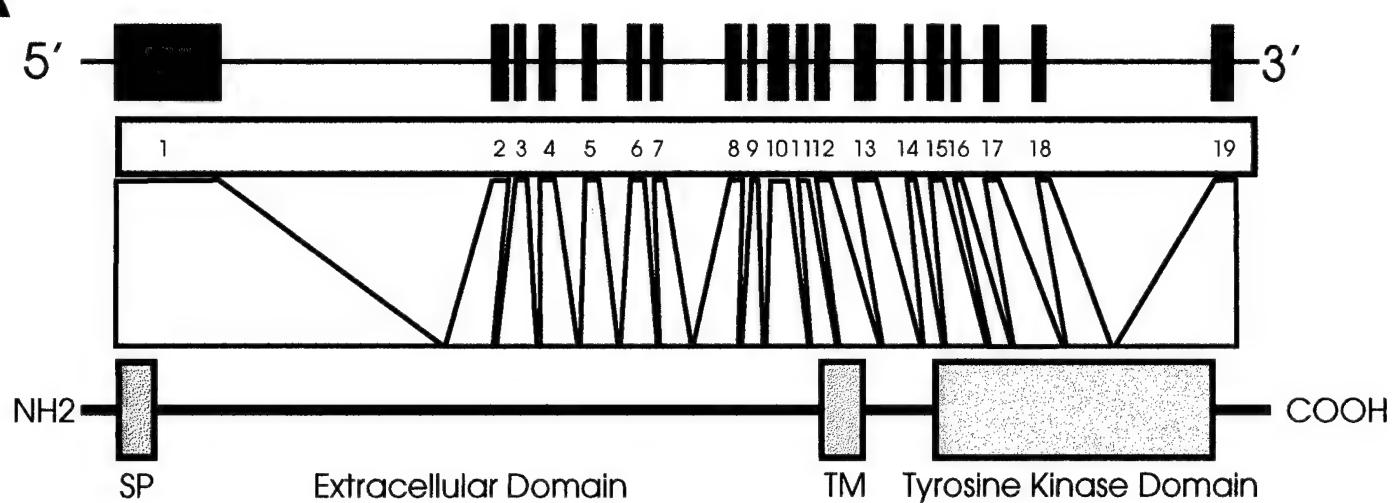
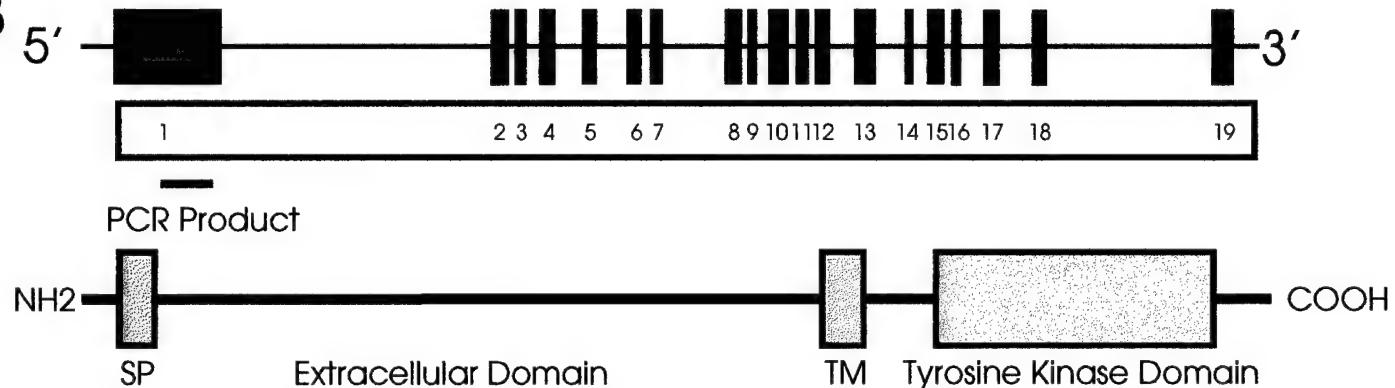


Figure 16

A



B



C

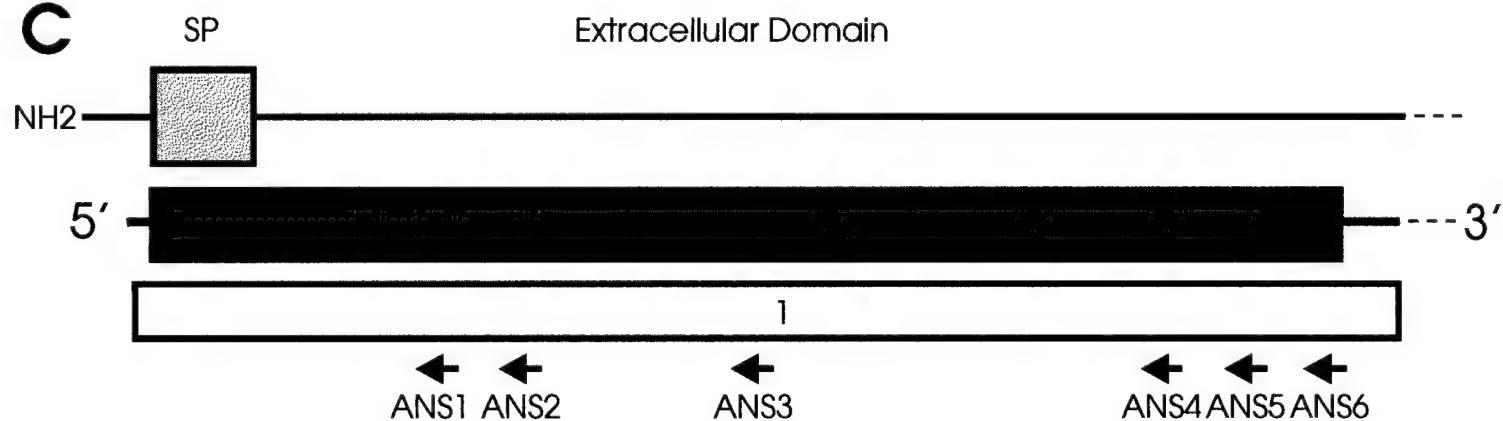


Figure 17



Figure 18

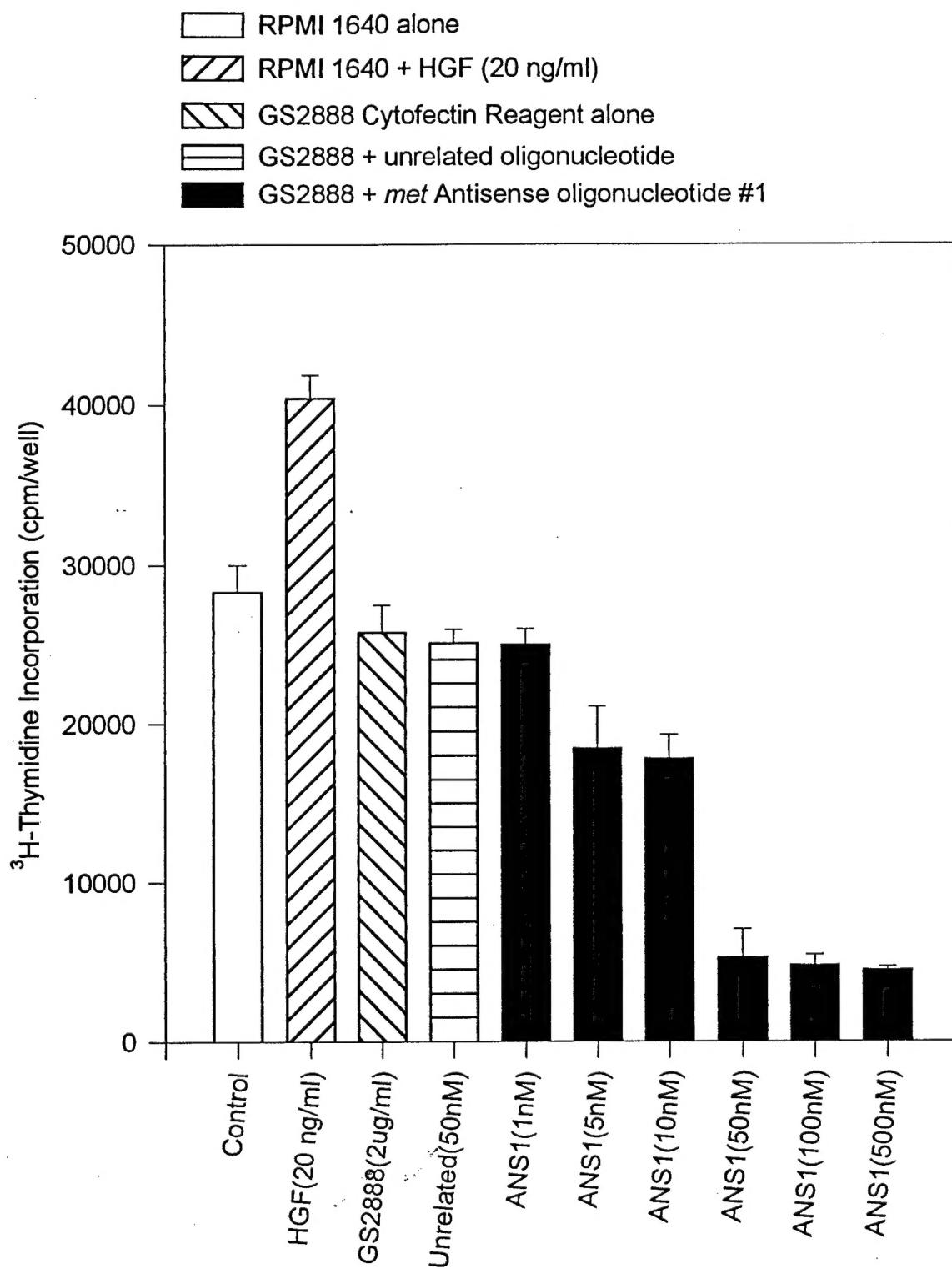
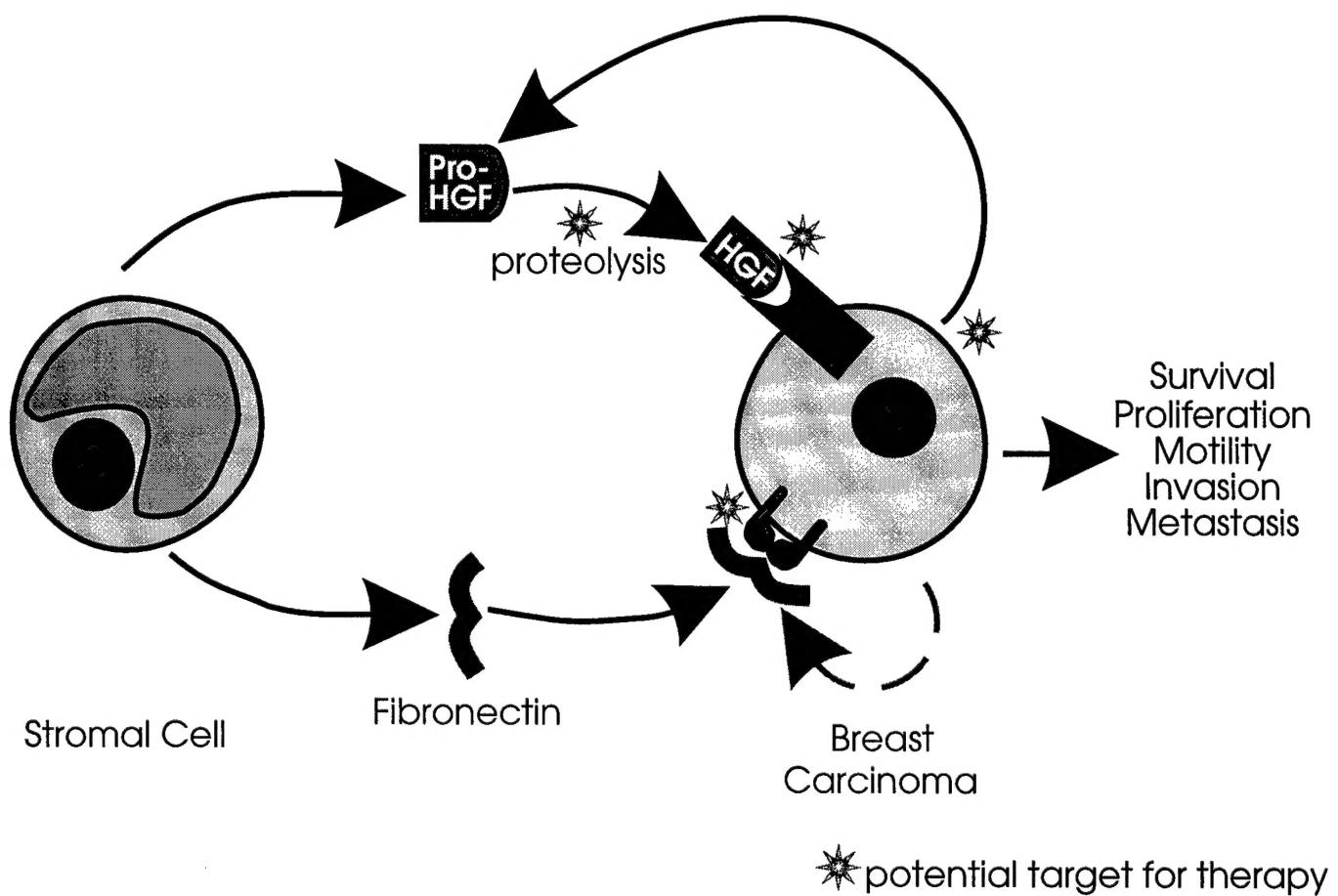


Figure 19: Model of stromal-tumor interactions



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June 18, 1997

Dr. Bruce Elliot
Cancer Research Laboratories
Third Floor, Botterell Hall
Queen's University
Kingston, Ontario
K7L 3N6

FAX #: 613-545-6830

Dear Bruce:

This letter is to affirm my interest in collaborating with you on the analysis of HGF and Met expression in human nonmalignant and malignant breast tissues.

As you know, my laboratory has developed procedures for separation of epithelial and stromal cell types from primary human breast tissues. I am pleased to provide such purified cell preparations for RT-PCR and protein analysis of HGF and Met in your system.

I look forward to interacting with you on this project.

Yours sincerely,

A handwritten signature in cursive ink, appearing to read "Joanne Emerman".

Joanne T. Emerman, Ph.D.
Professor

JTE/moc